Genes associated with variation in longevity and fecundity in sheep

by

Seung-Ok Byun

A thesis submitted in fulfilment of

the requirements for the Degree of Doctor of Philosophy

at

Lincoln University

New Zealand

2012

Abstract of a thesis submitted in fulfilment of the
requirements for the Degree of Doctor of Philosophy.

Genes associated with variation in longevity and fecundity in sheep

by

Seung-ok Byun

Longevity is an important trait in animal production systems because long-lived animals reduce the requirement to carry non-productive replacement livestock. Many factors affect animal longevity including animal fecundity and performance for whichever trait, or traits, is valuable in that production system. Longevity is therefore an economically important trait and should be a consideration in any breeding programme.

Little research has been undertaken on longevity in sheep. So-called "longevity genes" in other animal species have not been identified in sheep and/or effort has been primarily put into improving other sheep production traits. In this study, the aim was to identify sheep homologues for some putative longevity genes, including *IGF1R*, *FOXO3*, *CAST* and *TLR4*, to assess the extent of variation in these genes, and to ascertain whether variation in these genes is associated with longevity.

IGF1R is structurally conserved across species and its product is an evolutionarily conserved regulator of the insulin-like growth factor (IGF) signalling pathway from lower animals to mammals. Variation in *IGF1R* has been described in many species and associated with variation in longevity. In this study, a fragment of intron 2 and exon 3 of ovine *IGF1R* that encompasses the α subunits of the ligand-binding region, was screened for genetic variation. Three novel sequences (named *A*, *B* and *C*) were identified in this region of the gene. The three sequences were subsequently used to investigate associations with longevity.

FOXO3, a subgroup member of the Forkhead transcription factor family, plays an important role in mediating the effects of insulin and other growth factors on metabolism. Genetic variation in *FOXO3* has been associated with longevity in a number of species. In this study, the coding regions (two exons) of ovine *FOXO3* were firstly sequenced and identified. The entire exon 2 of ovine *FOXO3* was 1422 bp long and encompasses the C-terminus of the DNA-binding domain and a transcription activation domain. These are key regulators of transcription activity in the target genes that FOXO3 binds. Seven haplotypes with 10

nucleotide substitutions were identified across the 1422-bp exon 2 fragment. One of these substitutions was predicted to produce an amino acid change (p.M201V). The effect of the seven haplotypes (*A* to *G*) on sheep longevity was investigated.

CAST plays a role in protein-turnover and appears to be implicated in a range of phenotypes such as exercise-induced muscle injury, diabetes and degenerative neural diseases in humans. It has post-mortem effects on meat quality traits in livestock species and body weight traits in sheep. Recently, genetic variation in a non-coding region of *CAST* has been linked to longevity and fertility in dairy cattle, suggesting that this gene might play the same role in sheep. In this study, two highly polymorphic regions of ovine *CAST* were investigated revealing five sequences for exon 6 (*A* to *E*) and four sequences (*A* to *D*) for part of intron 12, respectively. Extended haplotype variation across the two regions was also investigated to better understand genetic diversity in ovine *CAST*. Nine haplotypes were defined across this extended region and four haplotypes were identified that suggested historical recombination events occurred within this gene. This intragenic recombination may make the discovery of phenotypic associations more challenging. Therefore, the five sequences (*A* to *E*) defined in the exon 6 of ovine *CAST* were further investigated in the context of sheep longevity.

TLR4 is a receptor for lipopolysaccharide (LPS) found on Gram-negative bacteria as well as a number of other endogenous ligands that drive immune responses to pathogens. In mammals, an optimized immune system plays an important role in good health and longevity. Variation in *TLR4* has been shown to be associated with various infectious and age-related diseases. In this study, seven sequences (*A* to *G)* were identified for exon 3 of ovine *TLR4* which encodes an important ligand-binding site, and these sequences were investigated and assessed for their effect on longevity.

Using the PCR-SSCP method, 1826 sheep, from six different breeds and 36 different stud breeding flocks, were genotyped for the variation described above in *IGF1R*, *FOXO3*, *CAST* and *TLR4*. The age of these sheep varied from 2 to 16 years old and fecundity data (the number of lambs raised per each year of their productive life) was collected for many of the ewes to investigate the relationship between longevity and fecundity. A general linear mixed model (GLMM) in SPSS version 17 (SPSS Science Inc., Chicago, IL, USA) was used to assess the effects of the variation in *IGF1R*, *FOXO3*, *CAST* and *TLR4*.

Genetic association study has revealed that genetic variation in both *IGF1R* and *FOXO3* had a significant association with sheep longevity, but genetic variation in *CAST* and *TLR4* had no significant effect on sheep longevity across breeds and flocks. Ovine *IGF1R C* was a significantly associated with an increase in age of about half a year (breed correction, $P = 0.009$ and flock correction, $P = 0.024$), while the *D* haplotype of *FOXO3*, which carries the

amino acid substitution p.M201V, was associated with a decrease in age of about five months (breed correction, $P = 0.006$ and flock correction, $P = 0.034$). The presence of *TLR4 A* and *TLR4 C* was associated or was trending to an association with fecundity when the model was corrected for 'breed' (1.64 ± 0.03, $P = 0.004$) and 'flock' (1.75 ± 0.02, $P = 0.066$), respectively. The Spearman's correlation coefficient between longevity and fecundity in the sheep studied was -0.248 ($P<0.001$), suggesting a weak negative interaction between these traits.

Keywords: Longevity, fecundity, insulin-like growth factor 1 (IGF1R), forkhead box class O 3 (FOXO3), calpastatin (CAST), toll-like receptor 4 (TLR4), sheep, genetic selection

Publications and Conference presentations arising from this thesis

Papers:

Byun, S.O., Zhou, H., Forrest, R.H., Frampton, C.M., & Hickford, J.G. (2012). An association between life-span and variation in IGF1R in sheep. *Journal of Animal Science*, Accepted.

Byun, S.O., Zhou, H., & Hickford, J.G. (2011). Characterisation of genetic variation in the Forkhead box class O3 (FOXO3) in sheep. *DNA and Cell Biology*, 30(7), 449-452.

Byun, S.O., Zhou, H., Frampton, C.M., & Hickford, J.G. (2010) No association between variation in the ovine calpastatin gene and either longevity or fertility in sheep. *Animal Genetics*, 41(2), 223-224.

Byun, S.O., Zhou, H., & Hickford, J.G. (2009). Development of a simple typing method for the ovine Toll-like receptor 4 gene. *Veterinary Immunology and Immunopathology*, 130(3-4), 272-274.

Byun, S.O., Fang, Q., Zhou, H., & Hickford, J.G.(2009). An effective method for silver-staining DNA in large numbers of polyacrylamide gels. *Analytical Biochemistry*, 385(1), 174-175.

Byun, S.O., Zhou, H., & Hickford, J.G.(2009). Haplotypic diversity within the ovine calpastatin (CAST) gene. *Molecular Biotechnology*, 41(2), 133-137.

Byun, S.O., Zhou, H., Forrest, R.H., Frampton, C.M., & Hickford, J.G. (2008). Association of the ovine calpastatin gene with birth weight and growth rate to weaning. *Animal Genetics*, 39(5), 572-573.

Zhou, H., Byun, S.O., Frampton, C.M., Bickerstaffe, R., & Hickford, J.G. (2008). Lack of association between *CAST* SNPs and meat tenderness in sheep. *Animal Genetics*, 39(3), 331-332.

Byun, S.O., Zhou, H., & Hickford, J.G. (2008). Polymorphism of the ovine insulin-like growth factor 1 receptor (IGF1R) gene. *Molecular and Cellular Probes*, 22(2), 131-132.

Conferences:

The Coopworth Breed Society Conference 20-22 May, 2008 at Palmerston North, New Zealand: Byun S.O. (2008) Genetic control of longevity and fertility, birth weight in Coopworth sheep.

The Coopworth Breed Society Conference 19-21 May 2009 at Lincoln University, New Zealand: Byun S.O. (2009) Genes associated with variation in longevity and fertility in sheep.

Lincoln University PG conference 29-30 August 2008 at Lincoln University, New Zealand: Byun S.O. (2008) Effect of variation in the ovine CAST gene on birth weight.

NZSBMB (New Zealand Society for Biochemistry and Molecular Biology) mini conference 21 September 2009 at Lincoln University: Byun S.O. (2009) A significant association with sheep longevity for the ovine IGF1R gene.

Sequences submitted to the NCBI GeneBank:

Ovine calpastatin gene, D sequence, intron 12: Accession number EF669476

Ovine toll-like receptor 4 gene, E sequence, exon 3: Accession number EU786152

Ovine toll-like receptor 4 gene, F sequence, exon 3: Accession number EU786153

Ovine toll-like receptor 4 gene, G sequence, exon 3: Accession number EU786154

Ovine insulin-like growth factor 1 gene, A sequence, exon 3: Accession number EF669473

Ovine insulin-like growth factor 1 gene, B sequence, exon 3: Accession number EF669474

Ovine insulin-like growth factor 1 gene, C sequence, exon 3: Accession number EF669475

Ovine forkhead box 3 gene, A sequence, exon 2: Accession number GQ995521

Ovine forkhead box 3 gene, B sequence, exon 2: Accession number GQ995522

Ovine forkhead box 3 gene, C sequence, exon 2: Accession number GQ995523

Ovine forkhead box 3 gene, D sequence, exon 2: Accession number GQ995524

Ovine forkhead box 3 gene, E sequence, exon 2: Accession number GQ995525

Ovine forkhead box 3 gene, F sequence, exon 2: Accession number GQ995526

Ovine forkhead box 3 gene, G sequence, exon 2: Accession number GQ995527

Ovine forkhead box 3 gene, the entire coding sequence: Accession number JQ894783

Acknowledgements

Studying towards my PhD has been a major turning point in my life. It has been, at times, an emotional, but overall a satisfying, eye opening and character building journey. Many people's patience, support and encouragement helped bring this thesis to completion. I would like to express my thanks to everyone that contributed, directly or indirectly, to this thesis.

First of all, I would like to express my deepest respect and most sincere gratitude to my supervisor Associate Professor Jon Hickford. He gave me guidance, understanding and lots of constructive advice that inspired me to continue and persist with this PhD study. Almost everything I know about animal genetics, I owe to him. Without his support and encouragement, this accomplishment would have never been fulfilled.

I would like to thank my co-supervisor Dr. Huitong Zhou. It has been an honour to be his first PhD student. He has taught me how to perform quality experiments and how to write academic papers.

My thanks also go to my co-supervisor Dr. Victoria Metcalf who kindly evaluated my writing.

This research was funded by the Coopworth society. I thank all those who provided financial support, and those who were responsible for awarding the following scholarships; Lincoln University Doctoral scholarship in 2007, the Macmillan Brown Agricultural Research scholarship in 2010 and 2011, the Heaton Rhodes scholarship in 2011 and the Syd Bodmin scholarship in 2011. Without generous financial support of all of these groups, it would not have been possible for me to complete this PhD study.

For helping me with the statistical analyses of my data, I owe thanks to Associate Professor Rachel Forrest and Associate Professor Chris Frampton.

I have also been fortunate to work with a great group of friends, staff and PG fellows at Lincoln University. These include Chris Logan, Jan Haldane, Alison Hind, Andrea Hogan, Caitriona Cameron, Freeman, Hua, Sajee, Jane, Yang, Wei, Grant, Kim, Amanda, Robbie and Sabrina.

Thanks to all the farmers who helped me with collecting samples.

Many thanks to mom, dad, sisters, brothers and my husband's family, who have always been there for me no matter what. I will never forget all of the unconditional love you have given me.

Finally I am thankful to God for bringing a very special husband into my life.

Table of Contents

List of Tables

List of Figures

Abbreviations

α	alpha	**GH**	growth hormone
β	beta	**IGF1R**	insulin-like growth factor 1 receptor
µg	microgram	**M**	molar
µL	microlitre	**MAPK**	mitogen-activated protein kinase
µm	micrometre	**mg**	milligram
µM	micromolar	**mL**	Millilitre
A	adenine	**mM**	millimolar
ANOVA	analysis/analyses of variance	**MHC**	major histocompatibility complex
AKT	protein kinase B	**ng**	nanogram
bp	base pair	**nm**	nanometre
C	cytosine	**nt**	nucleotide
CAST	calpastatin	**N-terminal**	amino-terminal
C. elegans	*Caenorhabditis elegans*	**PI3K**	phosphoinositide 3-kinase
cDNA	complementary DNA	**PKA**	protein kinase A
CNS	central nervous system	**PKC**	protein kinase C
C-terminal	carboxyl-terminal	**rpm**	revolutions per minute
CR	caloric restriction	**r**	correlation coefficient
D. melanogaster	*Drosophila melanogaster*	**r^2**	coefficient of determination
dNTP	deoxyribonucleoside triphosphate	***S. cerevisiae***	*Saccharomyces cerevisiae*
E. coli	*Escherichia coli*	**T**	thymine
EDTA	ethylenediaminetetraacetic acid	**TLR4**	toll-like receptor 4
FOXO3	forkhead box class O 3	**TM**	transmembrane
g	gravity	**Tris**	tris(hydroxylethyl) aminomethane
G	guanine	**UV**	ultra violet
GLMM	general linear mixed model	**V**	volt

Amino acid residue abbreviations

1-letter	3-letter	Amino acid	Side chain polarity	Side chain charge at pH7.4
A	Ala	alanine	nonpolar	neutral
R	Arg	arginine	polar	positive
N	Asn	asparagine	polar	neutral
D	Asp	aspartic acid	polar	negative
C	Cys	cysteine	nonpolar	neutral
E	Glu	glutamic acid	polar	negative
Q	Gln	glutamine	polar	neutral
G	Gly	glycine	nonpolar	neutral
H	His	histidine	polar	positive, neutral
I	Ile	isoleucine	nonpolar	neutral
L	Leu	leucine	nonpolar	neutral
K	Lys	lysine	polar	positive
M	Met	methionine	nonpolar	neutral
F	Phe	phenylalanine	nonpolar	neutral
P	Pro	proline	nonpolar	neutral
S	Ser	serine	polar	neutral
T	Thr	threonine	polar	neutral
W	Trp	tryptophan	nonpolar	neutral
Y	Tyr	tyrosine	polar	neutral
V	Val	valine	nonpolar	neutral

Chapter 1
Literature review

1.1 Introduction

Productive longevity in sheep is normally defined as the length of their productive life, or the amount of time that sheep spends producing (VanRaden & Klaaskate, 1993). In ewe flocks, it can reflect the ability of an individual ewe to delay being culled for having low productivity or succumbing to illness. Maintaining un-productive or under-productive ewes is economically inefficient; hence longevity plays a major role in the economics of sheep production. With increased longevity in a flock, it is possible to increase the life-time number of lambs born per ewe, thus making more lambs available for sale and without the need to retain juvenile stock to ultimately replace that ewe. Increased longevity in a flock also has another positive economic impact. Long-lived ewes do not need to be culled if meat prices are low and can instead be retained until the market situation improves and a higher value for them can be realised.

Despite the value of longevity in animal production system, there has been little research into identifying longevity genes in livestock. What is more, longevity studies in other species reveal that it is a complicated trait and potentially controlled by a large number of different and complex mechanisms. It is however heritable (Ricklefs & Cadena, 2008) and while several longevity genes have been described in other mammals (Rattan, 2006), investigation has been limited in the livestock industries. This is typically because effort has been primarily put into improving other production traits.

The viability of New Zealand's sheep industry is dependent on maintaining, if not enhancing its productivity. This can in part be achieved through the selective breeding of livestock to produce animals that are optimised for productivity in the system. To assist in animal breeding, Estimated Breeding Values (EBV's) have been calculated for many economically valuable traits and these are then used in breeding programs to improve the accuracy of animal evaluation (Van der Werf, 2007).

There are several limitations to this method as estimated breeding values cannot be easily calculated for some traits, especially those that are hard to measure. Among these "difficult to measure" traits is longevity, because it is impossible to measure on young

animals and therefore it cannot be bred for directly, or rapidly. Typically multiple generations of progeny have been produced before the trait becomes obvious in the older individuals.

Recent, advances in molecular genetics and statistical methodology have contributed to the identification of key genes in livestock and estimation of their effects on animal physiology. Knowledge of the action of these genes is now being applied to improving animal production (Williams, 2005).

If genetic selection by either method (EBVs or molecular genetics) is well managed, it is a cost-effective way of improving productivity and in a way that is both cumulative and permanent. As an example, the use of a quantitative genetic approach to breeding in the New Zealand dairy industry has resulted in a considerable improvement in key milk production traits (Harris, 2005; Harris & Kolver, 2001), while in the New Zealand sheep industry there is now widespread use of genetic selection for various traits using the services of Sheep Improvement Limited (SIL), including the calculation of EBV's for the number of lambs born, growth rate and carcass characteristics (Blair & Garrick, 2007).

In cattle production, there has been some progress in increasing longevity by means of genetic selection based on the use of EBV's. In addition, it was recently reported that there was an association between genetic variation in the bovine calpastatin gene (*CAST*) and longevity and fertility in dairy cattle (Garcia et al., 2006).

Given the genetic similarity between cattle and sheep and the conservation of many metabolic systems across the animal kingdom, it may therefore be possible to identify specific longevity genes in sheep and genes that could ultimately be used to improve productivity.

There are also emerging examples of the use of molecular genetics in improving production and disease resistance traits in livestock (see Appendix A). Molecular genetics has allowed us to identify genes that are associated with reproductive traits in cattle, goats, pigs and sheep (see Appendix B). To date emphasis has primarily been on the identification of SNPs that mark production traits, or on nucleotide changes in coding regions, but there is increasing evidence that non-coding regions of genes also explain variation in some production traits (e.g., 3'UTR variation in myostatin).

While improving longevity is desirable, the relationship between longevity and fertility is both intriguing and worthy of investigation in livestock. This relationship has been studied in various organisms and in general, longevity or life-span reflects good health, which also

positively influences both fertility and consequently fecundity (Ducrocq & Sölkner, 1998). Although there is less information on whether the relationship between life-time reproductive success and longevity is passed down through generations, longevity exhibits such a strong correlation with life-time performance that there is a strong potential for the inheritance of genetic factors that affect life-span.

However, one theory, called the "trade-off theory" (Holliday, 2006) suggests there is a negative relationship between longevity and fertility. According to this theory enhanced longevity comes at the expense of reduced fertility. Thus, fertility may be negatively influenced by potential longevity genes and *vice versa*. In this respect it is notable that in some livestock industries, there have been reports of declining fertility with age (Dobson et al., 2007), especially as these industries have focused on selection for increasing production traits. While improving longevity is desirable, the relationship between longevity and fertility is potentially antagonistic and both are accordingly worthy of investigation in livestock.

Studies in model organisms such as the flat worm *Caenorhabditis elegans* (*C. elegans*) (Kenyon, 2011), the fruit fly *Drosophila melanogaster* (*D. melanogaster*) (Clancy et al., 2001; Giannakou & Partridge, 2007) and the Ames and Snell dwarf mice (Bartke & Brown-Borg, 2004), have implicated, genes that affect metabolism, energy homeostasis, stress responses, and cell maintenance and repair in the regulation of longevity. The underlying processes appear to have been conserved through evolution and several biological pathways that affect aging and longevity in model organisms, also appear to affect longevity in other species.

In this study, a number of genes that have been described in other species as affecting longevity were identified in sheep and the relationship between variation in those genes and variation in both longevity and fecundity was investigated in a large population. The genes studied were the Insulin-like Growth Factor 1 Receptor gene (*IGF1R*), Forkhead box class O3 gene (*FOXO3*), Calpastatin gene (*CAST*) and Toll-Like Receptor 4 gene (*TLR4*).

1.2 Sheep production

Sheep are multi-purpose animals and provide meat, wool, milk and skin. Almost all sheep can be classified based on their ability to produce a particular product, or a combination of these products. The relative economic importance of these products varies from country to country. In the hotter parts of the Middle East, and generally in tropical climates, hair as

opposed to wool sheep predominate. This is understandable, as a heavy wool fleece is a handicap in very high temperatures. On the other hand, wool producing sheep are favoured in temperate and cold regions, where wool not only has a protective function, but also a considerable commercial value.

Sheep meat can be produced from both milk and wool types of sheep. Table 1.1 summarises world sheep meat production with Asia and Africa producing 67% of world sheep meat by volume. However, the average per capita production and consumption of sheep meat is highest in Oceania and countries in this region underpin the international trade in sheep meat (Table 1.1). In this region, New Zealand and Australia are major sheep meat exporting countries, accounting for over 90% of world export trade (source: major exporting countries of sheep meat in 2009, United Nations). Together they provide large quantities of sheep meat for consumption in the European Union (EU) and South West Asia.

Historically the sheep meat industry was developed as a by-product of the wool industry. For example in New Zealand the early 1900's, the majority of income from sheep was from the sale of wool. Since then changes in the wool industry have heavily influenced the direction of sheep production with the main productive value in New Zealand now being the sale of lamb. Wool production has been in a very deep economic crisis since the end of World War II, in part because of the reduction in wool use for military uniforms (Jones, 2004). In addition, by the mid-1960s, synthetic fibres were less expensive than wool, and synthetic/natural fibres blends were more attractive to consumers. Wool is however still economically important to many sheep farmers, textile manufacturers, retailers and the garment/fashion industry.

Internationally, Australia is the largest supplier of apparel wool, with two-thirds of the wool sold on the world market originating from that country (source from ABARE report). Major markets for Australian wool include China, Italy, India, Taiwan, the Czech Republic, the Slovak Republic, France and Germany.

New Zealand is the world's second-largest wool producer and exporter (MAF SONZAF report). More than 90% of the wool production is exported as fibre. New Zealand is the leading producer of coarser or strong wool, which is used primarily for interior textiles such as carpets, blankets, upholstery and yarn. China accounts for about 25% of New Zealand's wool exports. Other export markets include the United Kingdom, India, Italy and Australia.

Table 1.1. World sheep meat, milk, wool and skin production in 2009.

Continent	Sheep Meat Tonne, (%)	Sheep Milk Tonne, (%)	Greasy Wool Tonne, (%)	Sheepskin Tonne, (%)
Asia	**4294 (52%)**	**4074 (44%)**	**851 (41%)**	**1026 (53%)**
(China)	(2090)	(1150)	(364)	(326)
Africa	**1260 (15%)**	**1988 (21.5%)**	**222 (11%)**	**213 (11%)**
America	**411 (5%)**	**38 (0.5%)**	**165 (8%)**	**92 (5%)**
(South America)	(247)	(38)	(147)	(72)
Oceania	**1136 (14%)**	–	**550 (27%)**	**285 (15%)**
(Australia)	(658)		(371)	(144)
(New Zealand)	(478)		(179)	(140)
Europe	**1138 (14%)**	**3145 (34%)**	**257 (13%)**	**304 (16%)**
(UK)	(302)	-	(65)	(68)
(Greece)	(89)	(780)	(7)	(18)
(France)	(83)	(253)	(8)	(11)
(Turkey)	(262)	(734)	(40)	(46)
(Iran)	(350)	(577)	(74)	(58)
Total	**8240 (100%)**	**9245 (100%)**	**2045 (100%)**	**1920 (100%)**

Sources: FAOSTAT (Food and Agriculture Organization of the United Nations)

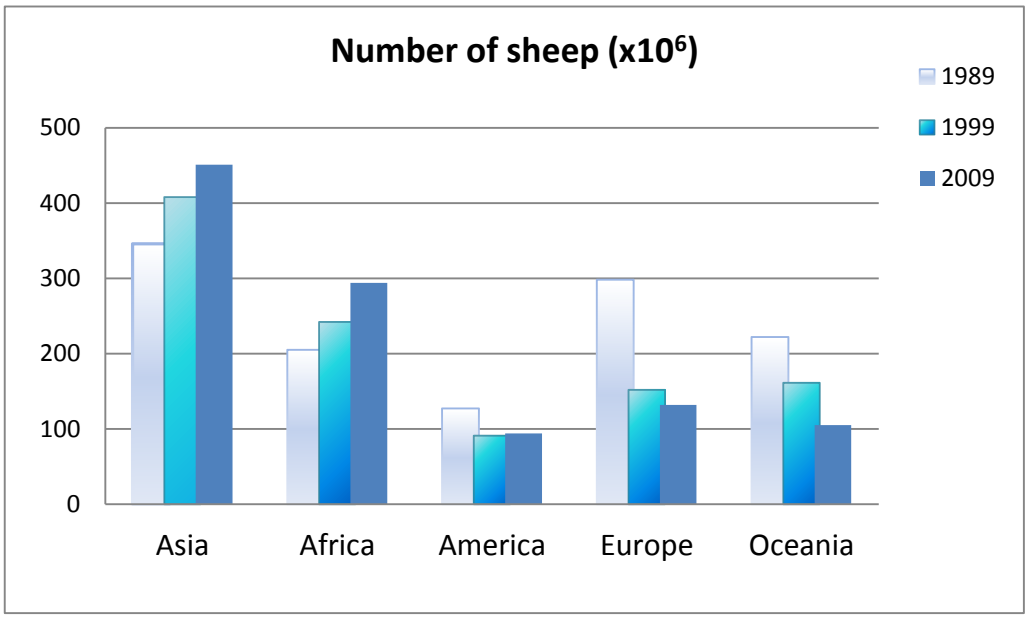


Figure 1.1. World sheep population.

Sources: FAOSTAT (Food and Agriculture Organization of the United Nations)

Sheep milk contains almost twice the concentration of solids when compared to cow or goats milk. The production of this milk, which is mainly used for cheese, is an important consideration in a number of countries (e.g., Turkey, France and Iran). Asia and Europe together produce 78% of the total milk production from sheep, but on a per capita basis, milk production in Europe exceeds that of any other continent (Table 1.1).

Sheepskin is usually a by-product of meat production and reflects the number of animals slaughtered for meat production in the various areas of the world. It is used for making sheepskin leather products and soft wool-lined clothing or coverings, including gloves, automobile seat covers and childcare applications such as cot, pram, and child car seat covers. Sheepskins are mainly produced in Asia and Africa. These two continents together produce 63% of the total world production (Table 1.1). For some sheep breeds, such as the Karakul, skin production is the primary product and these sheep are often bred for this purpose (Pascal, 2011). By-product sheepskins constitute an important source of export income in Oceania and South America.

Dual-purpose sheep have been developed for both meat and wool production with the capacity for high levels of production under appropriate environmental and management systems. In New Zealand, dual-purpose types of sheep such as the NZ Romney, Coopworth and Perendale are common and the production of both meat and wool is a major industry. Although sheep numbers continue to fall in NZ (Figure 1.1), it remains the world's largest exporter of sheep meat and cross-bred (strong) wool (http://www.stats.govt.nz).

There are a number of challenges facing the New Zealand sheep farmer, such as changing land-use patterns, climate change and the desire for greenhouse gas mitigation, and all of which will potentially impact on future sheep farming profitability. The cost of sheep management has increased, including the cost of disease control, labour and other input costs, while the income from meat and wool especially, has decreased in the last two decades. This has had a strong negative influence on sheep numbers in New Zealand.

World demand is now directed towards producing meat alone and less so wool in many places. This has caused the structure of the sheep industry to change. To overcome these changes in the market for sheep products and to overcome other challenges, the New Zealand sheep industry needs to transform itself into a more efficient and competitive entity.

Variation in sheep productivity can be due to both genetic and environmental influences. Genetic improvement in sheep productivity is founded in understanding the biology of adaptation and its relationship with productivity. This requires good estimates of

both the genetic and phenotypic characteristics of production. The effective selection of more productive sheep using genetic approaches will assist ensure continued high quality outputs are produced.

The New Zealand sheep industry has already shown a great improvement in sheep productivity, but new genetic approaches to improving sheep productivity may provide new opportunity for the industry.

1.2.1 The effect of life-span on sheep production systems

Longevity in livestock is typically a desirable trait and for several reasons. It reflects many things including ongoing good health and prolonged fertility. These characteristics are rewarded when selecting stock, as farmers require replacements that are at least as productive as the stock they are replacing.

Sheep are however susceptible to many diseases and parasites, and losses to death are high relative to other livestock. As a result, sheep farming requires more careful selection for breeding stock than other livestock production system.

Young sheep or lambs have a high mortality that is both variable and can range up to 80% of individuals dying. It averages 5 to 30% in New Zealand (MAF biosecurity & animal welfare NZ). After 2 years of age, mortality reduces and in general, sheep can live to over 10 years of age with individual ewes living up to 19 years of age. Rams typically survive to 14 years of age (Simmons & Ekarius, 2001).

Attainment of these ages in typical production systems is however rare. Typically, ewe longevity is reduced by the decisions made by farmers as they select animals to be culled based on their health, productivity and reproductive performance. Accordingly, the average life-span of a ewe in a commercial sheep flock is six to seven years before she is replaced. If the productive life of a ewe could however be increased to 10 years, then the life-time earning capacity of that ewe would increase and the cost of replacing her would decrease.

As the cost of replacing ewes is related to the cost of producing feeding and managing the replacements, the effect of having longer-lived ewes would be to reduce the number of replacements required. This could improve the efficiency of sheep production. This concept is illustrated in Figure 1.2.

Since the phenotype of longevity can be measured, albeit only after the fact, and given that the trait is under genetic control and hence is heritable, then it should therefore be possible to breed more long-lived sheep. That stated, it is unlikely the improvement will be

that spectacular, as the trait is both complex and affected strongly by environmental factors such as disease. What is more, a conventional approach to breeding through selecting long-lived animals will be manifestly slow, as longevity by its very nature, takes a lot of time to manifest.

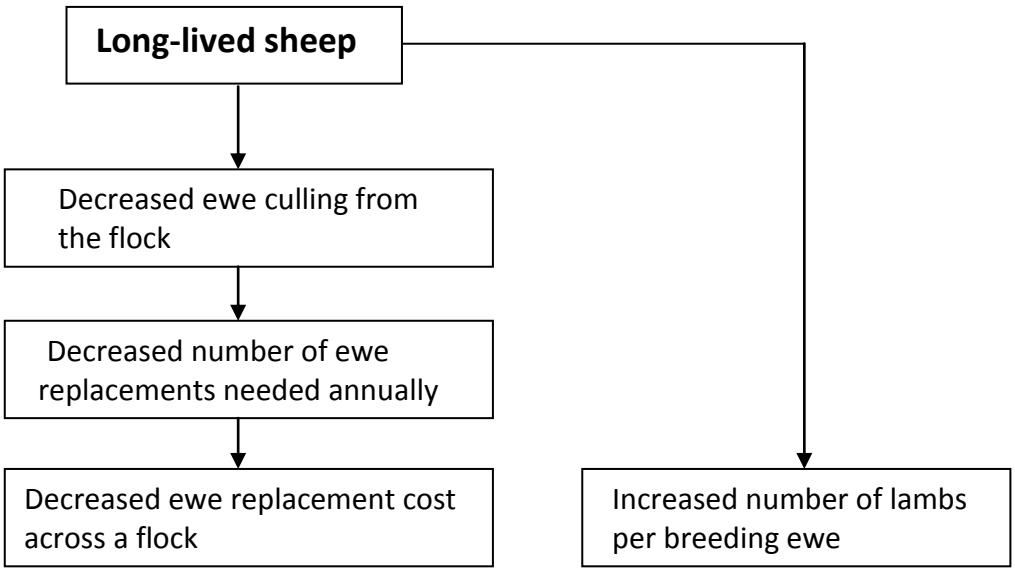


Figure 1.2. The value of longevity in sheep.

1.2.2 Measurements of longevity and reasons for culling sheep in production systems

Ducrocq & Sölkner (1998) defined two types of production longevity in livestock: "real" longevity and "functional" longevity. Real longevity is independent of productivity and is related to natural survival. However, functional longevity is related to an animal's productivity and depends on the ability of the animal to avoid culling for reasons such as low-fertility and disease susceptibility. According to this definition, functional rather than real longevity is the trait which should be selected for in a production system.

To maintain functional longevity in a productive ewe flock, sheep need to be replaced every year to maintain numbers. This is because ewes die of both natural and unexplained causes. They are also culled for various reasons including; udder health, tooth wear and loss, and because of the presence of disease (See table 1.2 for more detail on culling) (Nugent & Jenkins, 1992). In general, in New Zealand, 15-20% of a flock will be replaced or culled annually (MAF biosecurity & animal welfare NZ, 2010). Functional longevity is therefore composed of several components.

Sheep culling can be for two general reasons. Voluntary culling is usually for low performance (e.g., poor producer), and these animals may also accrue expenditure on animal health, whereas involuntary culling results from death, often due to misadventure or other environmental challenge. Losses from both can be a major financial cost to sheep production.

With voluntary culling the decision to replace any given ewe is mainly based on economic considerations (e.g., the breeder expects a greater profit from replacing the ewe than by keeping her in the flock). Whether to cull or not to cull a ewe depends not only on individual factors (e.g., health, fecundity), but also on flock factors (e.g., variation in flock size, availability of replacements, lamb and adult sheep meat markets, etc.). If an improvement in longevity was achievable it may allow the manager or farmer to place greater emphasis on culling sheep for low productivity.

Table 1.2. Reasons for culling sheep. Sheep farmers can improve their stock by making sound culling decisions. A reasonable culling decision should focus on traits that will maximise sheep production. As longevity reflects overall performance and good health, a longevity trait would be a useful addition to making effective selection decisions

Reason for culling	**Manifestation or trait of importance**
Poor reproduction	Lambing rate (singles/twins/triplets)
	Litter size and weight, occurrence of infertility, reduced fecundity or birth problems
	Poor lamb birth weight, weaning weights, poor ewe milk production,
	Udder soundness, uterine prolapse
Poor production	Carcass yield and quality, growth rate
	Wool traits such as staple length, fleece weight, fineness
Diseases susceptibility	Scrapie, footrot, internal parasites, infectious disease
Physical problem	Tooth wear/loss, lack of physical soundness, poor eyesight, lameness, body condition
To obtain genetic improvement	Genetic progress in meat and wool production traits
Other economic factors	Feeding cost, health care costs, sheep market price

1.3 The biology of aging and longevity

Aging in animal species appears to be influenced by several evolutionarily conserved metabolic signalling pathways and environmental factors such as nutrient availability and temperature. Growth and reproduction are also metabolically expensive and they create additional "costs" that contribute to aging (Brown-Borg, 2007). Most animals have the capacity to balance energy between growth, reproduction and the maintenance of somatic tissue (Takahashi et al., 2000). This maximises the likelihood of survival for the offspring when born.

Aging is associated with a decline in metabolic factors and this contributes to the accumulation of deleterious changes (Rattan, 2004). For example, metabolic factors from various biological signalling pathways and other regulatory proteins affect maintenance and repair mechanisms, and hence unbalanced control in these pathways can lead to the accumulation of damaged macromolecules (Breusing & Grune, 2008). Such molecular damage can appear as oxidized, misfolded, cross-linked or aggregated macromolecules, which are structurally abnormal and therefore cannot function properly. These ultimately affect other biological activities.

There are also several mechanisms that are considered to underlie the primary aging process and may contribute to age-related changes in adaptive responses. These mechanisms are involved in cellular aging (Miller, 2009), oxidative stress (Kregel & Zhang, 2007), mitochondrial energy production (Lenaz et al., 2000) and DNA damage in stem cell activity (Shenghui et al., 2009; Slijepcevic, 2008). All of these things can interact to influence the aging of an entire organism.

In many organisms from yeast to humans, the molecular, cellular and physiological aspects of aging are regulated by genetic factors (Finch & Ruvkun, 2001). Research has revealed conserved genetic systems associated with metabolism (Puca et al., 2008), protein regulation (Rattan, 2010) and immunity (Ostan et al., 2008), and that these can regulate the aging process in a variety of species primarily through genetically-derived in the various pathways involved (Glotov & Baranov, 2007).

For example, in the nematode *C. elegans*, aging-regulating genes have been identified and these can have both pro- and anti-aging effects (Antebi, 2007; Kenyon, 2005). Genes involved in the insulin-like growth factor 1 (IGF1) pathway and protein synthesis promote the aging process, while genes associated with cellular degradative pathways such as proteolytic

enzyme systems, slow the aging process. These pro- and anti-aging genes also significantly affect life-span in response to biological and environmental conditions, including caloric restriction and oxidative stress stimuli (Figure 1.3). The biological aging process therefore could be caused by a combination of several factors, including accumulation of cellular damage, environmental factors, and pro- and anti-aging factors (Figure 1.3).

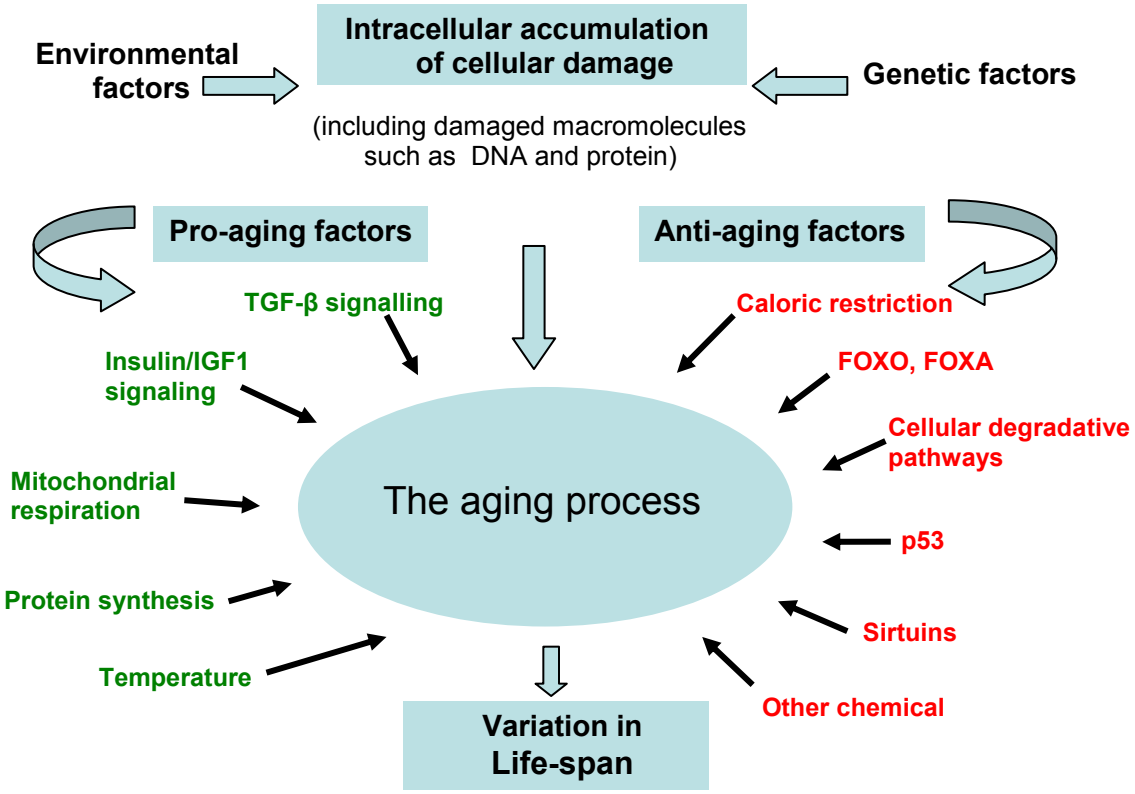


Figure 1.3. Regulation of the biological aging process. During aging, there is an accumulation of damaged macromolecules, including DNA and proteins. Although aging is a multi-factorial process, it is apparent that different factors activate some common, highly conserved molecular pathways which limit stem cell function, organ maintenance and regeneration. The pro-aging factors (green) accelerate the aging progress while the anti-aging factors (red) delay the aging process. The molecular mechanisms for maintenance and repair are also of crucial importance, and damage to these maintenance and repair pathways can increase the aging process. Adapted from Vellai (2009)

The aging process is biologically complex. A variety of experimental procedures have been used to examine changes in the cells, tissues or organs during the aging of various animal species (Balcombe & Sinclair, 2001). This has led to the proposition at a variety of theories of aging, and these are based on different aspects of biology at the cellular, systemic and molecular levels.

There are two basic types of aging theory that can be described broadly as the "programmed" and "error" theories (Platt, 1979). According to the programmed theories, aging depends on biological clocks regulating the timetable of the life-span through the stages of growth, development, maturity and old age. Programmed regulation would depend on genes sequentially switching on and off signals to the nervous, endocrine and immune systems. These systems are ultimately responsible for the maintenance of homeostasis and for activation of defense responses. In contrast, the error theories identify environmental insults to living organisms that induce progressive damage at various levels such as mitochondrial DNA damage, oxygen free-radical accumulation and the inappropriate cross-linking of proteins. While this basic classification is useful, it can be further refined into more precise groupings based on mechanism.

Table 1.3 summarises a classification system for the main mechanisms of aging, including evolutionary mechanisms (e.g., mutation accumulation, somatic cell maintenance), molecular mechanisms (e.g., variation in the expression of genes), cellular mechanisms (e.g., accumulated oxidative damage) and systemic mechanisms (e.g., endocrine shifts) (Weinert & Timiras, 2003). These mechanisms overlap at various levels, such as when molecular events lead to cellular alterations, which then contribute to organ and systemic failure. If these affect reproduction they may have evolutionary implications. Each of these mechanisms will be described below in more detail to reveal common aspects of the underlying biology.

Table 1.3. Classification of biological mechanisms thought to be associated with aging.

Mechanism	Description
Evolutionary	
Mutation accumulation	• The accumulation of somatic mutation arising from genome instability in a single generation
Disposable soma	• Somatic cells are maintained only to ensure continued reproductive success
Antagonistic pleiotropy	• Genes beneficial at younger age become deleterious at older ages
Cellular	
Replicative senescence	• An increase in the frequency of senescent cells, resulting from loss of telomeres
Mitochondrial dysfunction	• Oxidative metabolism produces highly reactive free radicals that subsequently damage lipids, proteins and

	DNA
Wear-and-tear	• Accumulation of injuries and tissue/organ damage
Apoptosis	• Programmed cell death from genetic events
System	
Neuroendocrine	• Alteration in neuroendocrine control by glucose homeostasis
Immunological	• Decline of immune function with aging, results in increased incidence of infectious and inflammatory diseases
Molecular	
Gene regulation	• Aging is caused by changes in the expression of genes that regulate both development and aging
Somatic mutation	• Accumulation of molecular damage, primarily to DNA/genetic material

1.3.1 Evolutionary mechanisms of aging

The evolutionary theories of aging provide an explanation for aging based on natural selection, as opposed to describing metabolic mechanisms per se. They were first formulated in the 1940s based on the observation of patients with Huntington disease, a genetic disorder of the central nervous system (Haldane, 1941). It was noted that the disease remained in the population even though it should be selected against. Its ongoing presence was explained by the late age of onset (30-40 years), which allowed a disease carrier to reproduce before dying, and thus pass on the faulty gene. There was therefore a lack of natural selection against the disease.

This observation inspired the mutation accumulation theory of aging, which suggests that mutations may accumulate in the population if they have little or no effect prior to reproduction, but that these mutations could ultimately lead to disease and death.

The concept of an evolutionary "trade-off" was proposed to explain the balance between maintaining somatic cells and facilitating reproduction (Holliday, 2006). The balance between somatic maintenance and reproduction would depend on the life strategy and ecological niche of any given species. Somatic maintenance or longevity would therefore have a cost and the balance of resources invested in longevity versus reproductive fitness would determine life-span.

While it might be possible to select for an increased total intake of energy, this does not avoid the problem of how best to divide the resources available between longevity and reproduction. The problem of the optimal allocation of resources becomes a central issue in life-history and particular attention has to be paid to the at times high cost of reproduction, as may be the case for those animals that breed rarely, or have few offspring.

This "trade-off" theory underpins both the "disposable soma theory" and the "antagonistic pleiotropy theory" (Weinert & Timiras, 2003). The "disposable soma theory" of aging suggests that the somatic organism is effectively maintained only for reproductive success and after that it is disposable, while the "antagonistic pleiotropy theory" suggests that genes that improve the likelihood of reproductive success, may ultimately be a cost post-reproductively to the aging organism.

The antagonism between reproduction and longevity explained by "the trade-off" theory is supported by experiments in which the limitation of reproduction by the destruction of germ line cells, can extend life-span in both *Drosophila melanogaster* (Sgrò & Partridge, 1999) and *Caenorhabditis elegans* (Arantes-Oliveira et al., 2002). This suggests that animals cannot have both higher rates of reproductive success and an extended life-span.

1.3.2 The cellular mechanisms of aging

Cellular aging occurs within cells and it is explained by replicative senescence, mitochondrial dysfunction, "wear and tear" and apoptosis. When cells accumulate damage at the cellular level they can stop functioning properly. This could damage their chromosomes, affect gene expression and hence, cellular aging could ultimately affect longevity.

Replicative senescence is a specific type of cellular aging that is associated with the loss of telomeres (Shawi & Autexier, 2008). The telomeres are repetitive DNA elements at the end of chromosomes and appear to protect the chromosomal DNA every time a cell divides. With each cell division, the very end of a chromosome cannot be copied as there is no DNA to act as a template for a primer to initiate replication of the lagging strand. When the telomere becomes too short, essential parts of the chromosomal DNA might not be replicated and this may contribute to cellular death. Although there is considerable variation in telomere length between individual animals within a species, shortened telomeres are nevertheless associated with cell age.

Mitochondria act as the "power-house" of a cell. The electron transport chain occurs in mitochondria and in this process electrons are passed between different molecules, with each

translation producing chemical energy (Lenaz et al., 2000). Oxygen is involved in the final position of the electron transport chain and occasionally electrons in the chain interact incorrectly with oxygen and produce oxygen in free radical forms. These free radicals are very reactive, and if cells continuously produce free radicals, then this can damage and eventually kill the cell.

With aging, the efficiency of mitochondrial function and energy production declines. This can lead to further mitochondrial dysfunction, disruption of cellular energy production and accelerated cellular aging (Trifunovic & Larsson, 2008). Evidence is accumulating that mitochondrial dysfunction underlies many common age-related disease, including Parkinson disease, Alzheimer disease, heart disease, and numerous genetic conditions (Simon & Johns, 1999; Trifunovic & Larsson, 2008)

The "Wear and Tear" theory, developed by August Weismann (1834-1914) is a cellular theory. It states that toxins in our environment and diet; such as excess sugar, caffeine and alcohol, and physical and emotional stresses cause damage of the cells, tissues and organs. The damage begins at the level of molecules within a cell. Eventually, the cells in the organs, such as the liver, stomach, kidney and skin are worn down by the progressively accumulated damage. Once they wear down, they cannot function correctly and that results in aging.

Apoptosis is a type of death for a cell in the body. It is a programmed cell death, meaning that when a cell gets old, weak or unneeded, it is deliberately disposed of. Apoptosis happens continuously in a variety of organisms, as the cells are deliberately killed and replaced. Normally, apoptosis is a natural and necessary process for the body. However, if apoptosis happens too often, or not often enough, then this causes problems. Some aging studies in human have shown that with age, a significant amount of apoptosis occurs, causing damage to skeletal (Pollack et al., 2002; Sandri & Carraro, 1999) and cardiac cells (Pollack et al., 2002) and that this is directly related to conditions such as osteoporosis and chronic heart failure.

The cell-cycle is tightly regulated, only a few tissue types have cells that are constantly dividing (e.g., skin, mucosa), whereas other cells enter a state of senescence, whereby they no longer grow and divide. Normally, in adult tissue however, there is a delicate balance between apoptosis and cell-cycle turnover, producing a steady state (Medema & Macurek, 2011). During the progression through the phases of the cell-cycle: G1, S (DNA synthesis), G2, and M (mitosis), DNA is duplicated and the chromosomes are distributed evenly between

the two daughter cells. To maintain the integrity of cell-cycle progression, cells proceed through several pauses, or "checkpoints"(Vermeulen et al., 2003). At one checkpoint, late in G1, the cell either enters a quiescent phase (called G0), or commits to DNA synthesis and the rest of the cycle. A second checkpoint is located at the end of G2 phase, triggering the start of the M phase (Mitosis). DNA damage checkpoints occur at the G1/S and G2/M boundaries.

After DNA damage, the cell-cycle checkpoints are activated. Activated checkpoints pause the cell-cycle and give the cell time to repair the damage before continuing to divide. They are controlled by two master kinases, ATM (ataxia telangiectasia mutated kinase) and ATR (ataxia telangiectasia and Rad3-related kinase) (Vermeulen et al., 2003). ATM and ATR phosphorylate several substrates, such as p53, the checkpoint kinases (Chk1 and Chk2) and regulate checkpoint signalling. ATM responds to DNA double-strand breaks and disruptions in chromatin structure (Bakkenist & Kastan, 2003), whereas ATR primarily responds to stalled replication forks.

If the DNA damage is irreparable, or too severe, activation of p53 may not only arrest the cell-cycle, but also initiate the process of apoptosis, which eliminates a cell whose DNA alterations could not be repaired (Reinhardt & Schumacher, 2012). Therefore, failure of DNA repair or checkpoint controls can lead to an accumulation of mutations and genomic instability, which in turn may lead to development of cancer (B. B. Zhou & Elledge, 2000).

Cells enter "senescence", a state of stable proliferative arrest, in response to variety of cellular stresses, including telomere dysfunction, aberrant oncogenic activation, DNA damage and oxidative stress (Bree et al., 2002; Hornsby, 2011). These stresses have a significant effect on cellular senescence. Such senescence cells are often dysregulated in cell-cycle checkpoints and apoptosis, resulting in premature aging, increased sensitivity to carcinogens and a correspondingly increased cancer risk.

1.3.3 System mechanisms of aging

In mammals, the neuroendocrine and immune systems play a key role in the response to external and internal stimuli, and are essential for the control and maintenance of the organ systems (Knight, 2000). In the system theories of aging, the aging process is related to the decline of the organ systems, and through failure of the regulatory system to maintain structure and function. When demand exceeds the capacity of the organ or organism to respond, pathology ensues.

The neuroendocrine theory proposes that aging is due to changes in neural and endocrine functions that are crucial for maintaining an optimal functional state for reproduction and survival, while also maintaining a response to environmental demands (Kirkwood, 2011). The primary hormone involved in the neuroendocrine system is insulin-like growth factor 1 (IGF1). IGF1 has an important role in both tissue maintenance and developmental processes (Martinelli et al., 2008). With increasing age there is a decline in IGF1 production in the liver and other tissues and the loss of IGF1 contributes to the general decline in protein synthesis, skeletal muscle mass, immune function, and cognitive ability in rodents, nonhuman primates and humans (Berryman et al., 2008).

In *C. elegans*, the IGF1 pathway constitutes a primordial neuroendocrine system, which integrates information from environmental stressors (Gerisch et al., 2001). The resulting integrated response plays an important role in monitoring metabolic and reproductive status and permits appropriate energy adjustments and ultimately, extend life-span. Thus, it may be assumed that this primitive neuroendocrine system has the capacity to coordinate various metabolic activities that affect longevity.

The immune system controls and eliminates foreign organisms and substances in the host body (Butcher & Lord, 2004). It has been suggested that maintaining effective immune function is important to having a long life (Pawelec, 2003). Immunity in the elderly is a crucial determinant of life-span, because without effective immunity susceptibility to disease including age-related diseases increases, and thus survival can be compromised.

The rate of aging is largely controlled by having an optimal immune system. Aging of the immune system (immunosenescence), is associated with decreased immunity or having a less functional immune system that leads to chronic inflammation (De Martinis et al., 2005). While, inflammation is a normal reaction to physiological and nonphysiological stressors such as infection, irritation and injury in young animals, immune cells can also be stimulated by age-related stressors and this can result in chronic stimulation. This can lead an expansion of memory cell numbers, a decrease in the number of naïve cells, and shrinkage of the reactive T-cell repertoire (Ginaldi et al., 2005). The decreased number of naïve cells may reduce the function of the immune system, resulting in an increase in the incidence of tumours and greater susceptibility to infection.

1.3.4 Molecular mechanisms of aging

Molecular mechanisms of aging explain that aging is primarily associated with differences in the genetic constitution of species (Teixeira & Guariento, 2010). Genes predetermine the maximum age by controlling the development of the organism, which controls the maximum life-span in that species. In these aging theories, genetic alterations such as changes in gene regulation and somatic mutation can accelerate the aging process.

Longevity studies have found that disruption of gene regulation mechanisms involved in stress-responses, metabolism and reproduction are significantly associated with the aging process in many species. The insulin-like growth 1 (IGF1) signalling genes are instrumental in this process and have been revealed to be important in regulating life-span in species as diverse as *C. elegans* (Golden & Melove, 2007), *D. melanogaster* (Paaby & Schmidt, 2009) and *M. musculus* (Liang et al., 2003). This suggests that the genetic control of life-span was already developed in the common ancestor of modern animals and that the subsequent evolution of life-span variation is underpinned by quantitative changes in the control of metabolism through IGF1 signalling.

Somatic mutation explains aging in terms of accumulation of mutations in the genome of somatic cells, that leads to cell senescence, cell death (apoptosis) or transformation, and loss of processes (Weinert & Timiras, 2003). Many of the mechanisms implicated in somatic mutation are shared with mechanisms involved in cellular aging.

1.3.5 Summary

Aging is considered a multifactorial process with interactions of genetics, chemistry, physiology and environment. As described above in any mechanisms have been proposed to explain the aging process in a variety of species. The most widely accepted theories describe the major causes of aging as mechanisms that operate at the evolutionary, cellular, systemic and molecular levels. The various theories explain what aging is, and why and how it happens. Typically, they describe the fundamental variability in longevity in different species. The various theories provide a framework for understanding mammalian aging, including aging in sheep.

Some of these theories of aging are linked, as the aging process entails a variety of phenomena within a complexity network. For example, there is a clear interrelationship between evolutionary theories and systemic theories. The evolutionary theories of aging are fundamental to the understanding of life-span by describing the balance between survival and

reproduction, and the metabolic processes that underpin them. However, given that metabolic performance is coordinated by systemic regulatory mechanisms, such as the endocrine and immune systems in organisms (Harman, 2004), then systemic effects must also be considered.

There may also be genetically determined evolutionally trade-offs between benefits to young organisms and their viability at older ages, suggesting that molecular theories of aging can be explained from an evolutionary view point. The molecular theories of aging are also inter-related to cellular theories of aging, as cells can age through the accumulation of DNA damage.

These overlapping theories of aging suggest that the emergence of a unified theory is still some time off.

1.4 The relationship between longevity and fertility

Metabolic rate is thought to be inversely proportional to maximum life-span (Demetrius, 2004) and it has been acknowledged for some time that a primary determinant of how long species live is the relative speed of their resting metabolism. Typically species that "live fast" will die young, while species with a slower metabolic rate live "slower" and longer. This notion was historically incorporated in to the rate of living theory (Pearl, 1928); in which it was proposed that "the duration of life" varies inversely as the rate of energy expenditure during its continuance. Based on "the duration of life" theory, the disposable soma theory was introduced, and this better explains the importance of balancing the cost of reproduction against the need to maintain the soma or organism as a whole. It suggests that maximum life-span is the result of optimisation of the metabolic trade-off between longevity and fertility (Kirkwood, 1977).

Nutrition, reproduction and life-span are closely interlinked in the trade-off theory. Specifically, in times when food is scarce, re-allocation of resources away from current reproduction to somatic maintenance might increase survival (Kirkwood & Holliday, 1979). This increase in survival would facilitate on increased likelihood of enduring to plentiful times, when increased reproduction is more profitable.

According to the disposable soma theory, trade-off is driven by the conflicting requirements for resources (Kirkwood & Holliday, 1979). Costly processes such as reproduction, somatic maintenance and repair, growth and movement compete for resources and it is impossible to maximize allocation to them all. "Trade-offs" must therefore be made.

Resources that are diverted into reproduction cannot also be used for somatic repair and maintenance, and a high reproduction rate will accordingly shorten life-span. According to this theory, there would be a negative relationship between longevity and fertility in any given species.

Caloric restriction (CR) has been associated with the trade-off theory. When nutrient intake is restricted, a reallocation of resources occurs from reproduction capacity into somatic protection. CR extends life-span and reduces fecundity in model organisms such as yeasts (Oliveira et al., 2008), nematodes (G. Walker et al., 2005), fruit flies (Partridge et al., 2005), rodents (Merry, 2005) and even humans (Everitt & Le Couteur, 2007).

Functionally, CR may promote longevity by a metabolic reprogramming which results in a transcriptional shift towards reduced energy metabolism and increased biosynthesis and turnover of proteins (R. M. Anderson & Weindruch, 2007). Some of the common and consistent effects of CR in rodents and humans (Weindruch, 1996) include lower fat mass, particularly visceral fat, lower circulating insulin and IGF1 concentrations, increased insulin sensitivity, lower mean body temperature, lower fat-free mass, lower sedentary energy expenditure (adjusted for fat-free mass), decreased levels of thyroid hormones and decreased oxidative stress (Pamplona & Barja, 2006).

The effect of CR on longevity is believed to be mediated by regulation of the IGF1 pathway. The IGF1 pathway is responsive to nutrients, growth factors, and the energy balance of an organism and regulates many metabolic mechanisms that modulate longevity and reproduction (Partridge & Gems, 2002). Mutations or treatments that decrease the levels, or effectiveness of IGF1, often result in an increase in longevity. Likewise, the IGF1 pathway increases life-span through the readjustment of the metabolic trade-off with reproduction. Overall the aging process might therefore be the result of the failure of maintenance mechanisms balancing reproduction and life-span via metabolic regulation.

1.5 Genetic control of longevity

Molecular and evolutionary theories suggest that a strong genetic component impacts on survival to an advanced age. The molecular theories suggest that genes that control the level of DNA damage and repair mechanisms regulate longevity (Hsieh & Yamane, 2008), while the evolutionary theories suggest that reproductive cost is traded off against the cost of longevity. There is also evidence that genes have evolved to detect and respond to changes in

the environment (e.g., food supply), suggesting biological evolution contributes to longevity (Ljubuncic & Reznick, 2009).

The evolutionary theories of aging suggest that animals maintain themselves using a wide range of mechanisms and that these consume a considerable proportion of their total metabolic activity (Ricklefs, 1998). These biological pathways include nutrient metabolism pathways, cell maintenance mechanisms and differentiation pathways. All of these mechanisms are regulated by gene-encoded proteins, making longevity potentially heritable (Browner et al., 2004; Kirkwood, 2002).

There are several direct pieces of evidence that longevity is controlled by genetic factors. Variation in the life-span both within and between species strongly supports the concept that, to a large extent, the rate of aging is under genetic control. For example, in mice, there are significant differences in life-span between different inbred strains of laboratory mice, and even when these strains are in an identical environment (Ferguson et al., 2008). In humans, the life-span of monozygotic twins is statistically closer than life-span in dizygotic twins (Hjelmborg et al., 2006).

Studies have also revealed an association between genes and longevity in model organisms such as *C. elegans*, *D. melanogaster*, *S. cerevisiae* and *M. musculus* (Kennedy, 2008). These organisms provide many advantages for studying longevity, most notably because of their relatively short life-spans, rapid reproduction, well-characterised biology and completely sequenced genomes. These organisms have allowed rapid progress in the discovery of genetic factors and pathways that affect aging.

The first pathway of this kind that was identified was the evolutionarily conserved insulin/IGF1 signal-transduction pathway. In *C. elegans*, mutations in the *daf-2* and *age-1* genes, which are related to the mammalian insulin-like growth factor 1 receptor (IGF1R) and to the catalytic subunit of phosphatidylinositol-3-kinase (PI3 kinase) respectively, both lead to increased life-span (Friedman & Johnson, 1988; Kenyon et al., 1993; Larsen et al., 1995; Morris et al., 1996). In subsequent studies, similar effects were observed for other genes in the IGF1 pathway, and in other pathways involved in metabolism and physiological processes that regulate stress resistance, fertility and genomic maintenance (Christensen et al., 2006; Vijg & Suh, 2004).

Collectively, these studies in lower organisms suggest that specific genes may also affect the rate of aging in higher organisms, including mammals.

1.5.1 Genes implicated in longevity

The identification and characterisation of the genes involved in various biological processes is important in the study of longevity. Genes that potentially affect longevity are found associated with metabolism, mitochondrial function, immune response, heat-shock responses and others physiological responses. In mammals, several studies have reported that single nucleotide polymorphisms (SNPs) in a variety of genes, have an association with longevity (Guarente & Kenyon, 2000; Perls, 2002; Tatar et al., 2003).

Analysis of the literature that describes specific longevity genes reveals that they are involved in a range of biochemical pathways and act as kinases, kinase receptors, transcription factors, DNA helicases, membrane glucosidases, GTP-binding protein–coupled receptors and chaperones. Some are involved in cell-cycle checkpoint pathways. All of these activities contribute to maintaining the biological mechanisms of normal aging in mammals, including sheep.

There is a challenge in classifying the various genes that have been associated with longevity. This is not least because so many genes have been linked to the trait. The most prominent longevity genes include Angiotension 1-Converting Enzyme (*ACE*) (Rahmutula et al., 2002), Angiotensinogen (*AGT*) (Tan et al., 2001), Apolipoprotein A (*APOA*) (Garasto et al., 2003), Apolipoprotein E (*APOE*) (Atzmon et al., 2006), Calpastatin (*CAST*) (Barendse et al., 2007), Carboxypeptidase B2 (*CPB2*) (Reiner et al., 2005), Cholesteryl Ester Transfer Protein (*CETP*) (Barzilai et al., 2003), Coagulation Factor VII (*F7*) (Tan et al., 2001), Forkhead Box O3 (*FOXO3*) (Flachsbart et al., 2009), Growth Hormone 1 (*GH1*) (Van Heemst et al., 2005), Glutathione S-Transferase Theta (*GSTT1*) (Taioli et al., 2001), Human Leukocyte Antigen (*HLA*) (Akisaka et al., 1997), Heme Oxygenase (*HMOX1*) (Yamaya et al., 2003), Heat Shock 70kDa Protein 1A (*HSPA1A*) (Altomare et al., 2003), Interferon Gamma (*IFNG*) (Lio et al., 2002a), Insulin-like Growth Factor 1 Receptor (*IGF1R*) (Bonafè et al., 2003), Interleukin 6 (*IL 6*) (Bonafè et al., 2001), Insulin Receptor (*INSR*) (Kojima et al., 2004), Integrin Beta 3 (*ITGB3*) (Hessner et al., 2001), Klotho (*KL*) (Arking et al., 2002), Major Histocompatibility Complex (*MHC*) (Lio et al., 2002b), 5-Methyetrahydrofolate-Homocysteine Methyltransferase (*MTR*) (Linnebank et al., 2005), Transforming Growth Factor, Beta 1 (*TGFB1*) (Carrieri et al., 2004), Toll-Like Receptor 4 (*TLR 4*) (Candore et al., 2006a) and Tumour Necrosis Factor (*TNF*) (Soto-Vega et al., 2005).

It is likely that many more genes associated with aging have yet to be discovered, further complicating the challenge of classification. Up until now, there appears to be no

widely accepted classification system for the various longevity genes, as longevity is a complex trait and the various genes interact with other genes and the environment. However, there are suggested classifications, including the various categories of genes that are outlined below (Browner et al., 2004):

- **Genes involved in the regulation of DNA repair and nuclear structure and function:** Genes associated with Werner syndrome (*WRN*) and Lamin A (*LMNA*). When mutated these genes cause progeroid syndromes.

- **Genes involved in the regulation of telomere length:** Shorter telomeres have been associated with shorter survival. Genes associated with Human Telomerase RNA (*hTR*), Dyskeratosis Congenital 1 (*DKC1*).

- **Genes that affect stress resistance and oxidative damage:** Longevity and stress resistance are closely linked. Oxidative stress actually constitutes a defined hypothesis of aging in that macromolecule oxidative damage accumulates with age and tends to be associated with longevity. Genes associated with Superoxide Dismutases (*SOD*), Insulin Receptor (*IR*), Insulin-like Growth Factor1 Receptor (*IGF1R*).

- **Genes encoded by mitochondrial DNA, reactive oxygen species:** Mitochondria are the major source of reactive oxygen species, also known as free radicals, within a cell. Mutations in mitochondrial DNA reduce production of ATP and lead to cell death and aging.

- **Genes that mediate the effects of caloric restriction:** The responses to caloric restriction and other forms of cellular stress trigger various mechanisms, which increase life-span, including activation of the sirtuin pathways. Sirtuins are deacetylase enzymes that regulate gene expression. Genes associated with Silent Mating Type Information Regulator1 (*SIRT1*).

- **Genes that regulate signalling by insulin and insulin-like growth factor-1 (IGF1) - like molecules:** The best studied longevity pathway involves insulin/IGF1 signalling. Reduced activity in this pathway is associated with prolonged life-span in several species. Genes associated with IGF1 signalling such as Insulin Receptor (*IR*), Insulin-like Growth Factor 1 Receptor (*IGF1R*), Forkhead Box O3 (*FOXO3*).

- **Genes involved in inflammation:** Genes that increase the inflammatory response to infectious organisms are associated with harmful effects, such as atherosclerosis. A common SNP (Asp299Gly) in Toll-like Receptor 4 (*TLR4*), which reduces the inflammatory response to bacterial infection, is associated with longevity. Other genes related to longevity are Interleukin 6 (*IL6*), C-Reactive Protein (*CRP*).

- **Genes that control protein degradation:** Age-related changes in protein-turnover show that especially intracellular protein degradation decreases with the aging process. Calpastatin (*CAST*) is one of the proteolytic systems which control protein degradation.

Choosing candidate genes that control longevity for study in sheep, is therefore potentially very challenging, especially as considerably less is known about the sheep genome than the genome of humans, mice and other well studied species.

However, most genes in the above categories have been conserved through evolution and have homologues with apparently similar functions across species. In this thesis, I have chosen to study genes that are both characterised to some extent in sheep and that are representative of at least some of the above groupings, and investigated as many longevity genes as possible. These genes are *IGF1R*, *FOXO3*, *CAST* and *TLR4*. While *IGF1R* and *FOXO3* are well known among the identified longevity genes, *CAST* and *TLR4* have relatively received little attention from researchers studying longevity.

1.5.1.1 The Insulin-like Growth Factor Receptor 1 gene (IGF1R)

The endocrine system is a complex system of glands that produce hormones that influence almost every cell, organ, and function of the body. Among the many processes and activities regulated by hormones and hormone receptors, it has been demonstrated that they may affect both life-span and reproduction (Piper et al., 2008; Russell & Kahn, 2007; Taguchi & White, 2008; Tatar et al., 2003)

IGF1R is a hormone receptor and it is expressed by most organs and tissues. This receptor mediates the effects of IGF1, which is a polypeptide protein hormone similar in molecular structure to insulin. IGF1 plays an important role in carbohydrate and lipid metabolism, growth, survival and fertility in a variety of species. Studies of various animal models suggest that life-span may be affected by IGF1R (Barbieri et al., 2003).

Pioneering work by Kenyon et al. (1993) showed that genes in the IGF pathways are important in determining nematode life-span (Cynthia, 2001). This knowledge allowed subsequent research to target homologous genes in more complex model organisms like the fruit fly and mouse. The latter was the first mammalian model system. Principal IGF pathway components and their role in mammalian life-span regulation are now under wide-spread study, including IGF1R, which has become the focus of much attention.

IGF1R is originally known for its prominent role in the promotion of somatic growth and development in mammals and other vertebrates (Dupont & Holzenberger, 2003). Two different IGF1R mouse models (Baker et al., 1993; J. P. Liu et al., 1993; Selman & Withers, 2011) have shown IGF1R is required for normal growth and survival. These mice have either

introduced IGF1R mutations (Baker et al., 1993), or homozygous IGF1R null mutations (J. P. Liu et al., 1993). The former mice have increased neonatal death rates and reduced postnatal growth rates if they survive. They have smaller birth weights, at about 60% of normal controls and suffer from severe developmental delays. The latter mice die at birth from respiratory failure (Baker et al., 1993; J. P. Liu et al., 1993; Selman & Withers, 2011).

In contrast, a study by Holzenberger et al. (2001) has indicated that mice with partial IGF1R's are viable, but have postnatal growth retardation. It appears that as long as the IGF1R inactivation does not exceed 50%, then the effects on somatic growth remain subtle and the mice are healthy despite their mutation.

The control of cell growth and metabolism is critically important to survival. Studies in mice have shown that IGF1R is not only regulating growth, but also survival. In a recent longevity study using heterozygous knockout mice (*IGF1R*$^{+/-}$ mutants: mice with 50% of wild-type receptor levels); because null mutants are not viable, Holzenberger et al. (2003) have shown that heterozygous mice live on average 26% longer than their wild-type littermates. When the sexes are evaluated separately, heterozygous females live on average 33% longer than wild-type females, whereas male mutants live on average 16% longer. Heterozygous mice appear to have normal energy metabolism, nutrient uptakes, and levels of physical activity that are identical to that of their wild-type controls. Moreover, no change is observed in male and female fertility and reproduction. This finding supports the contention that IGF1R affects mammalian longevity.

1.5.1.2 The Forkhead box class O3 gene (FOXO3)

Mammalian FOXO3 regulates the transcription of target genes that act in the insulin/IGF1 pathway (Kloet & Burgering, 2011). It contains a highly conserved central DNA binding domain (the Forkhead Box) that binds the target genes via their FOXO response element, located in their promoters. FOXO3 binding not only results in increased transcription of the majority of the target genes, but it also can serve as a transcriptional repressor (Calnan & Brunet, 2008; van der Vos & Coffer, 2008).

FOXO3 functions as a key link in the IGF1 signalling pathway and is thought to influence both aging and longevity. The initial evidence that *FOXO3* might be a key downstream effecter of the PI3K-AKT pathway was derived from genetic studies in *C. elegans* (Mukhopadhyay et al., 2006). Inactivating mutations in the *C. elegans* insulin receptor (*daf-2*) or PI3K (*age-1*), resulted in up to 3-fold extended longevity. Importantly, this

life-span extension was reverted when the *C. elegans daf-16* was mutated. These genetic studies confirmed that the IGF1 signalling pathway is a major determinant of life-span, and mutation in *FOXO3* may therefore have negative effects on IGF1 signalling, as it controls gene expression of the pathway through direct binding to target DNA sites. This suggests that *FOXO3* may be an important candidate for genetic longevity studies in mammals.

Recent studies have indicated that polymorphism in the human *FOXO3* gene can influence longevity (Flachsbart et al., 2009; Willcox et al., 2008). This would suggest that the role of *FOXO3* in longevity is evolutionarily conserved, and hence that *FOXO3* may influence longevity in sheep.

FOXO3 also controls various biological functions, including cell-cycle progression (Medema et al., 2000; Tran et al., 2002), detoxification of reactive oxygen species (ROS) (Kops et al., 2002), repair of damaged DNA (Tran et al., 2002), apoptosis (Brunet et al., 1999; Dijkers et al., 2000), cell differentiation and glucose metabolism (Kashii et al., 2000) through activating or suppressing gene transcription. The ability to detoxify ROS and to repair damage is correlated with increased organismal longevity in many species (Kirkwood & Austad, 2000), and these additional functions of FOXO3 may be relevant to FOXO3's ability to control longevity.

FOXO3 has also been shown to play role in mammalian reproduction. An increasing number of studies has provided evidence that FOXO3 controls crucial steps in embryogenesis and is important for the development of ovarian follicles and reproductive organs (John et al., 2007). A recent study by Castrillon et al. (2003) has now confirmed a function for FOXO3 in the control of follicular development. In this work, FOXO3-null mice show abnormal ovary development, and are sterile.

Although FOXO3 acts as a key nuclear target of activation of IGF1 signalling pathways in a variety of species, no study focused on *FOXO3* has been undertaken in relation to longevity in any livestock species. In this thesis, FOXO3 will also be investigated for its association with longevity and fecundity

1.5.1.3 The Calpastatin gene (CAST)

The proper functioning of a cell requires structural proteins, enzymes, and regulatory proteins to be synthesised, used and degraded at appropriate times. The concentration of individual cellular proteins is determined by a balance between the rate of synthesis and rate of degradation (also called protein-turnover) (Cuervo & Dice, 1998), which in turn is

controlled by a series of regulated biochemical mechanisms (Hebert & Molinari, 2007). Protein-turnover is believed to decrease with aging in all organisms.

There is also an age-related increase in the concentration of abnormal proteins, and this is a common feature of aged cells (Rosenberger, 1991). The generation of abnormal proteins may be due to genetic mutation, failure of the protein to properly fold, or denaturation of the protein induced by various environmental stresses such as heat, oxidation, the present of heavy metals, and the effect of ultraviolet light exposure. Unfolded proteins are structurally unstable, and they may aggregate and precipitate (Glabe, 2008; Ono et al., 2009). If these abnormal proteins accumulate in the cells and tissues, they may interfere with intracellular transport mechanisms, hence disrupting normal cellular processes. The control of protein-turnover is therefore essential to cellular well-being.

Abnormal proteins, including excessive levels of particular proteins are normally broken down by the proteolytic degradation systems. Proteolytic systems are responsible for most intracellular protein-turnover and they are essential to ensure homeostasis and the controlled degradation of regulatory proteins that are necessary for cellular activities. Four proteolytic systems have been identified in eukaryotic cells: the ubiqutin-proteasome system, the calpain-calpastatin system, the lysosomal compartment, and the mitochondrial proteases (Liton et al., 2009). Impairment of these proteolytic systems is associated with several age-related neurodegenerative diseases (Alzheimer, Parkinson and Huntington diseases), cancer (Attaix et al., 1999) and autoimmune diseases (Martinez-Vicente et al., 2005; J. P. Taylor et al., 2002).

The calpain-calpastatin system is a major cellular proteolysis system and has been implicated in the aging process (Nixon et al., 1994). In general, this system regulates muscle growth by altering the relationship between protein-synthesis and protein-degradation, indicating once again its importance in the important regulation of protein-turnover (Hayashi et al., 1985; Klasing et al., 1987). Calpains also undertake specific proteolytic cleavages of several tissue-specific proteins, and therefore modify and control the function of these proteins (Goll et al., 2003).

Calpain activity is regulated by the specific endogenous inhibitor, calpastatin (CAST) (Goll et al., 2003). The calpain-calpastatin system is also modulated by intracellular calcium concentrations. Once calpains are activated, they are likely to play a role in degradation of certain membrane and cytoskeletal proteins (Figure 1.4) (Cuervo & Dice, 1998). However,

the calpains are usually present in an inactive form associated with CAST, suggesting that calpain activity is primarily regulated by CAST activity.

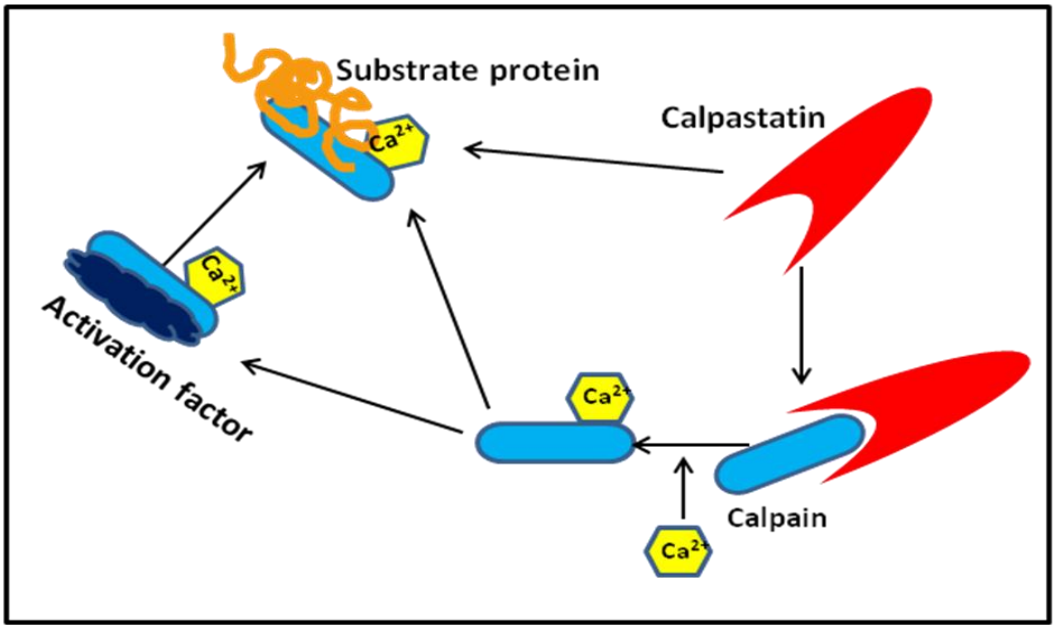


Figure 1.4. Protein degradation by the calpain-calpastatin system. Calpains are usually present in an inactive form associated with calpastatin, an intracellular inhibitor. After translocation to the cell membrane and limited autolysis, calpains are activated

Studies of CAST have revealed its role in various physiological and pathological functions such as differentiation of myoblasts (Dedieu et al., 2003) and adipocytes (Patel & Lane, 1999; Yajima et al., 2006), actin reorganization (Dedieu et al., 2004), apoptosis (Harwood et al., 2005) and cataract formation (Biswas et al., 2005).

In recent studies, CAST function has been reported to be importance in the aging process and age-related diseases including Alzheimer disease, diabetes, atherosclerosis and ischaemia (Ibrahim et al., 1994). These diseases are caused by the accumulation of certain peptides in the brain, pancreas and blood vessels. These studies suggest that CAST is likely to be linked to the aging process and probably by regulating calpain proteolytic activity (Baudry et al., 1986).

CAST may also regulate calpain activity in mammalian reproductive tissues and cells, as it is ubiquitously present in placenta (Takano et al., 2011), oocytes (Malcov et al., 1997), and testis (Li & Goldberg, 2000; Ravulapalli et al., 2009). A recent study in rat has suggested that CAST mediates rat oocyte activation and embryo development through the calpain-specific breakdown of α–spectrin, a cytoskeletal protein (Haim et al., 2006; Malcov et al.,

1997). This effect of the calpain-calpastatin system has also been observed in human oocytes (Ben-Aharon et al., 2005), placenta (Thompson et al., 2002) and testis (Rojas et al., 1999), suggesting that CAST activity plays a pivotal role in reproduction in mammals.

In livestock, CAST is known to be a regulator of post-mortem beef tenderisation and muscle proteolysis, and genetic variation in *CAST* is now used as a gene-marker for meat tenderness in cattle (Casas et al., 2006; Schenkel et al., 2006) and pigs (Ciobanu et al., 2004; Koćwin-Podsiadła et al., 2003). Although all these outcomes are of value to the livestock industries in improving meat quality, very little work has been done in examining the effects of *CAST* on longevity and fertility. A recent study reported that polymorphic variants of this gene are associated with longevity and fertility in dairy cattle (Garcia et al., 2006), further supporting the contention that this gene is associated with variation in both longevity and fertility.

1.5.1.4 The Toll-Like Receptor 4 gene (TLR4)

The major function of the immune system is to recognise foreign biological entities (antigens) and destroy or inactivate them either directly killing them, or by tagging them with very specific antibodies. The effectiveness of the immune system in most animals declines with age (Pawelec, 2003). Age-related loss of immune function, also called 'immunosenescence', is of importance, not only in terms of protection against infectious diseases (Hazlett & Wu, 2011), but also in several age-related chronic diseases such as Alzheimer disease, atherosclerosis, diabetes, cancer and some autoimmune diseases (Pawelec, 2003).

Studies in elderly humans and "old" mice have demonstrated that the innate immune system has a profound influence on immunosenescence (Gomez et al., 2005). Innate immunity is the first line of defense against pathogens as it activates immediately after the body encounters a pathogen. It includes the activity of neutrophils, dendritic cells (DCs), macrophages, natural killer (NK) cells and the complement system.

By comparison adaptive or acquired immunity involves the activation and expansion of lymphocytes populations. The adaptive immune response is antigen-specific and requires the recognition of specific non-self antigens during antigen presentation. The two parts of the immune system (innate and adaptive) are not independent, but are closely interlinked. Activated innate immunity not only mediates adaptive immunity, but also maintains acute and chronic inflammatory responses by controlling the level of inflammatory conditions (Larbi et

al., 2008; Pawelec, 2003) (Figure 1.5). Chronic inflammation is caused by elevated systemic inflammatory responses, and is a major driving force of age-related diseases (Vasto et al., 2007). It can be referred to as "inflamm-aging" (Franceschi et al., 2000).

The acute inflammatory response is a primary defense and protects an animals' body against bacteria, viruses, parasites and other invading organisms by releasing immune cells and chemical mediators, that maintain homeostasis in response to injury or infection (Libby, 2007). This inflammatory response should eradicate the infection and then subside to allow normal tissue to be rebuilt. However, if the acute inflammatory response persists for a prolonged period of time due to over-activation, (e.g., an increased sensitivity of inflammatory response or dysfunction of the innate immune system in the distinguishing between self and non-self in autoimmune disorders) (Gomez-Mejiba et al., 2009), the inflammatory response may become a chronic inflammatory response.

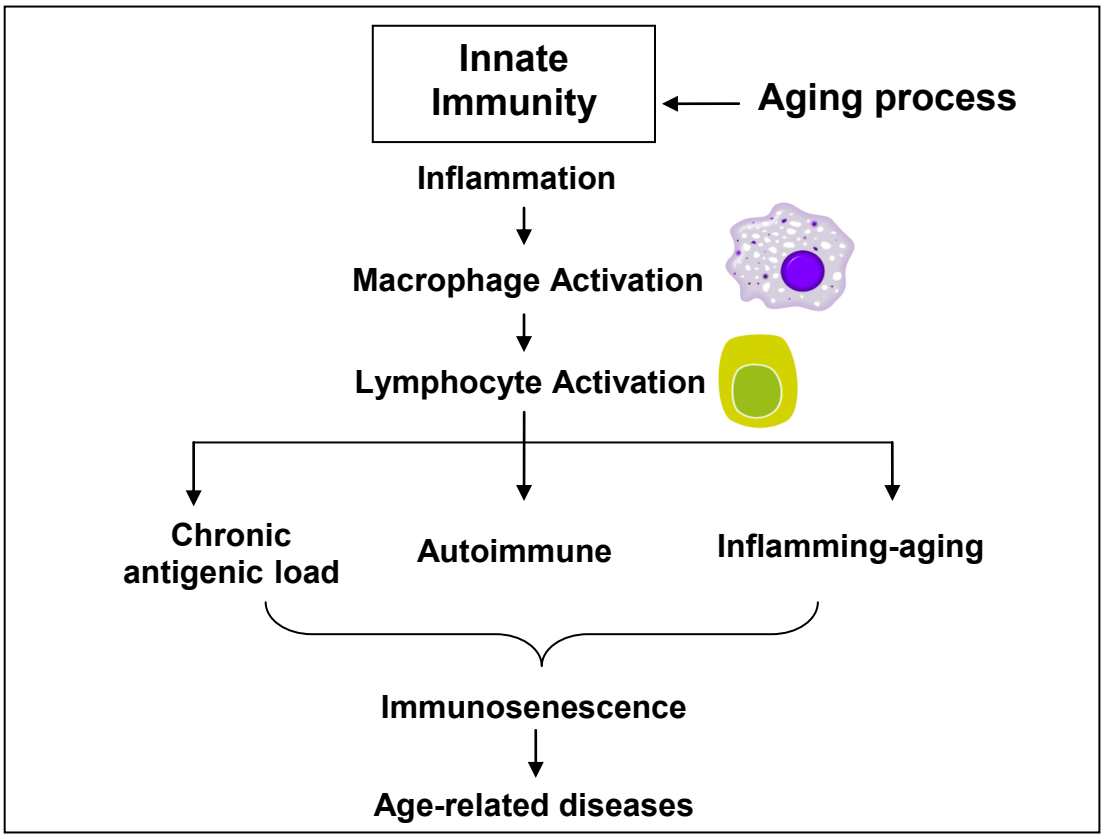


Figure 1.5. The effect of the aging process on the innate immune system. Adapted from Larbi et al. (2008)

The toll-like receptors (TLRs) are major constituents of innate immunity and play an essential role in inflammatory responses. TLRs recognise pathogen-associated molecular

patterns (PAMPs) on microorganisms. The PAMPs are unique to the microorganisms and not to the hosts and this allows discrimination between self and non-self. There are various groups of PAMPs and these include a wide variety of bacterial components such as lipids, carbohydrates, peptides and nucleic-acids (Kumar et al., 2009). Recognition of PAMPs by TLRs leads to a series of signalling pathways, resulting in innate immune responses and inflammation (Bauer et al., 2009).

Mammalian TLRs are classified into groups based on the types of PAMPs they recognise. To date, 13 TLRs have been described in mice and 11 in humans (Trinchieri & Sher, 2007). Of the known TLRs, Toll-like Receptor 4 (TLR4) recognises not only the lipopolysaccharides (LPS) of Gram-negative bacteria, but also other viral PAMPs and endogenous molecules, including the heat-shock proteins HSP60, HSP70 and GP96 (Beg, 2002; Beutler, 2000).

Excessive exposure to the PAMPs can cause antigenic overload and this can affect the innate immune system throughout life, leading to progressive activation of innate immunity and generation of inflammatory responses.

Most of the parameters influencing immunosenescence appear to be under genetic control (Candore et al., 2006) and it fits with the basic concepts of aging whereby what may be valuable in a younger organism, may become a cost in that organism as it ages. Accordingly, the innate immune system may be beneficial for reproduction and parenting by neutralizing infection agents, but it can be detrimental later in life by under-pinning chronic inflammation.

Single nucleotide polymorphisms (SNPs) in TLR4 have been described to be related to the incidence or progression of human inflammation-related diseases, such as asthma, atherosclerosis and rheumatoid arthritis, and also to cancer in humans (Achyut et al., 2007; Balistreri et al., 2009; L. C. Chen et al., 2007) and in mice (Pasterkamp et al., 2004). For example, human *TLR4* SNP +896A/G has been revealed to have an association with the development of cardiovascular disease.

Despite the recognition of a role for TLR4 variation in controlling longevity, little or no effort has been made to determine if *TLR4* variation will occur in sheep, or whether that impacts on their longevity.

1.6 Aim of this thesis

Sheep are expected to live up to 20 years and the average life-span of sheep outside of commercial flocks is 10 to 12 years (Simmons & Ekarius, 2001). In sheep production systems, the length of a sheep's productive life-time tends to be much less. This is because a ewe's productivity is usually highest between 3 and 6 years of age and begins to decline after the age 7. As a result, survival and longevity in commercial adult sheep typically depends on a farmer's culling decisions, and is based primarily on measures of health and production performance.

The replacement of breeding ewes poses a large cost to the New Zealand sheep industry, and inevitable wastage, as ewe replacements that do not "make the grade", need to be culled. This is often when their meat value has declined relative to the cost of feeding and maintaining those replacements. Having longer-lived sheep would reduce the requirement to carry non-productive replacements.

As described above longevity in animals is controlled by a large number of different biological pathways and it is a heritable trait. It can be influenced by many factors both environmental and genetic. From simple yeast to humans, genes have been identified that are implicated in determining longevity. These genes have many activities including being involved in hormone signaling pathways, metabolic pathways, proteolytic systems and immune responsiveness. Variation in these genes has also been shown to affect longevity in a variety of animal species, with both positive and negative impacts recorded. The gene associated with variation in longevity often share common functions across different animal species and thus they have the potential to provide insight into longevity in those species.

Longevity in animals can be influenced by many factors, both environmental and genetic. There will be genetic diversity within breeds and between breeds that might be important as regards their longevity. Therefore, sample collection is the first and the most important step in planning a study of sheep longevity. The main breeds chosen for study in this thesis were Coopworth and NZ Romney. However, those two breeds were not considered sufficiently diverse to be able to reliably describe diversity. Accordingly, a large number of ewes from many different flocks spread all over New Zealand were used. This not only creates genetic diversity but also allows variation in environmental factors to be better accommodated.

Methodologically, the single-stranded conformational polymorphism (SSCP) technique was used to determine genetic diversity in the sheep studied. This technique is ideal for studying a large number of genetic samples and at a low cost. SSCP enables large numbers of sheep to be rapidly screened for genetic diversity over larger regions of a gene. It does this more effectively than PCR- restriction fragment length polymorphism (RELP) or single nucleotide polymorphism (SNP) analysis and it can be done prior to the cost of cloning and sequencing amplicons to reveal nucleotide differences. Microsatellites analysis while useful for revealing regions of large variability and those regions that are useful for association analysis, can be less effective at finding functional gene variation especially if the microsatellites fall outside of the gene region and these are only in linkage disequilibrium with the functional in the gene.

Using the PCR-SSCP method, the aim of this thesis was to identify sheep homologous for the putative longevity genes *IGF1R*, *FOXO3*, *CAST* and *TLR4*, ascertain the nature and extent of variation in these genes and investigate what role they may have in controlling sheep longevity and fertility. If any genetic variation in these candidate genes is found to be significantly associated with sheep longevity, then it would provide options for further study and with a longer-term aim of developing gene-markers for longevity.

Chapter 2
The relationship between longevity and fecundity in sheep

2.1 Introduction

While improving longevity may be desirable in sheep, the potential for a relationship to exist between longevity and fecundity is worthy of investigation. This relationship has been studied in a variety of organisms and in general, longevity or life-span reflects good health which positively influences both fertility and fecundity (Ducrocq et al., 1988). However, it has been suggested that there is possibly a "trade-off", whereby an organism may have a limited amount of resources available for investment in reproduction, maintenance and somatic growth (Holliday, 2006) and this the cost of reproduction may negatively influence longevity and *vice versa*.

Recently, a relationship between longevity and fertility has been observed in the experimental nematode organism *C. elegans* (Kirkwood, 2005) and the fruit fly *D. melanogaster* (Schnebel & Grossfield, 1988*)*. In both species, the germ cells (that give rise to sperm and oocytes) influence the aging of the whole animal (Flatt et al., 2008; Hsin & Kenyon, 1999). If the germline precursor cells are removed in either the nematode (Hsin & Kenyon, 1999) or the fruit fly (Flatt et al., 2008), life-span is extended by 40-60%. These germline deleted organisms not only live longer than normal, but they also remain youthful and active for longer, suggesting that loss of the germline precursor cells extends life-span by slowing the animals' rate of aging.

Interestingly, removing the entire reproductive system of *C. elegans* does not extend life-span (Hsin & Kenyon, 1999), which argues against there being a simple reproductive trade-off. This suggests that a specific signal from the germ cells allows the animal to coordinate its rate of aging with the timing of reproduction.

Since reproduction is costly, but essential, it makes the task of surviving even more challenging, and primarily because of the energetic and nutritional requirements of pregnancy, lactation and parenthood. Pregnancy and lactation take energy and nutrients away from other processes including other physiological and metabolic functions. Accordingly, if a female has a higher number of offspring, this may negatively affect her health, especially at older ages, and ultimately reduce her longevity.

The idea, that there is a "cost of reproduction," has been put forward as an example of an evolutionary constraint, but this doesn't mean that there must always be a trade-off between reproduction and longevity. For example in some species, individuals die soon after mating or have shorter life-spans as a consequence of mating. In contrast, many examples of life-span extension with little or no inhibition of reproduction have also been confirmed (Kenyon, 2005). This illustrates the plasticity of aging and reproductive fitness in different species.

In humans, Westendrop & Kirkwood (1998) investigated pre-modern British aristocracy and reported a negative correlation between the number of progeny and female longevity, which they concluded, provided evidence that human life histories involve the "trade-off" between longevity and reproduction. However, a positive relationship between human fecundity and longevity has also been reported (Borgerhoj Mulder, 1988; Voland & Engel, 1989). It would appear that the "trade-off" is only for women who have considerably more than average number of children (Voland & Engel, 1989) and that modern medicine may have now affected the apparent trade-off.

In livestock production systems, the longevity of individuals within a herd or flock becomes a critical issue. For example, the fitness and longevity of dairy cows in herds has an impact on profitability (Coelho & Barbosa, 2006; Essl, 1998; Sewalem et al., 2006). There is also evidence that selection exclusively for production traits such as milk yield and fertility, may cause a correlated reduction in longevity (Essl, 1998; Wall et al., 2006). If the trade-off such as this exists in dairy cattle, it is therefore appropriate to investigate the relationship between longevity and fecundity in sheep.

Given the apparent potential for a trade-off between reproductive performance and longevity to exist in sheep, it is necessary to investigate the relationship between longevity and fecundity prior to investigating whether the former is under genetic control. If there is no negative relationship found between them, then long-lived ewes might provide both production quality and reproductive performance. This may enable genetic selection for improved longevity, without any loss of performance.

2.2 Materials and Methods

2.2.1 Details of the sheep to be studied

For the sheep longevity study, blood samples were collected over the years 2007-2009. Forty stud farms from all over New Zealand provided blood samples from ewes. These stud farms were chosen specifically because breeding sheep are frequently retained longer in studs than commercial sheep.

Farmers were asked supply the oldest ewes possible to assist in finding the genes that may affect longevity. For each sample the current age of the sheep in years was recorded. To compare between older and younger sheep, blood samples were also collected from two-tooth (two year old) replacement ewes on each farm. The geographical locations of the farms are shown in Table 2.1.

Table 2.1. Geographical region of the 40 farms studied.

No. of Farms	Region
8	Southland
9	Otago
6	Canterbury
4	Wanganui/Rangitikei/Manawatu
3	Central North Island
3	Waikato
2	East Coast
2	Wairarapa
1	Hawke's Bay
1	Auckland
1	Northland
Total 40	

The recording of lambing data was undertaken by the farmers and in the study group lambing occurred from late August to October. For each ewe a record was kept of how many lambs were raised to weaning each year.

Blood from a total of 1826 ewes was collected on FTA cards (Whatman BioScience, Middlesex, U.K.). Blood drops on FTA card were sampled from the ear of the sheep using electrical side-cutters. Details on sampling can be found in appendix C.

This study did not require ethical approval as ear-clipping is considered a standard sheep management practice. The blood was obtained from sheep of the Coopworth, Corriedale, NZ Romney, Merino, Polwarth, Hampshire, Texel and Kelso breeds. The main breeds were the Coopworth and NZ Romney. Farmers were willing to provide these different breeds for this study. The Coopworth breed was specially provided through collaboration with the Coopworth Sheep Society, hence why it is the main breed in this study. This breed is an internationally unique and a valuable genetic resource for finding genetic effects, because it is outbred.

A total of 40 farms were used to investigate the relationship between longevity and fecundity in this chapter, but some farms were excluded from the subsequent longevity and fecundity analyses for assessing genetic variation in the potential longevity genes (*IGF1R*, *FOXO3*, *CAST* and *TLR4*). These farms had small numbers of Hampshire and Texel sheep and this may have biased the analyses because of the potentially confounding effect of breed, sire, environment and management.

2.2.2 Sheep investigated to define variation in the potential longevity genes

The Gene-Marker Laboratory in Lincoln University provided a collection of blood samples to enable the development of the tests to screen for the identification of genetic variation in the IGF1R, FOXO3, CAST and TLR4 genes. These samples were randomly selected from wool and meat research projects and where of a variety of breeds, both gender and of variable age (see details in materials and methods section of chapter 3, 4, 5 and 6). As a consequence of the samples for longevity analysis having to be used at least four times with genotyping for the four different longevity genes, some of the samples were not enough to use screen for variation in the four genes. The longevity samples were collected over a two year period, which is another reason why samples from the Gene-Marker Laboratory were used for initially identifying genetic variation before all the longevity blood samples were collected.

2.2.3 Statistical analysis

Frequency data and basic descriptive statistics were calculated using SPSS version 17 (SPSS Science Inc., Chicago, IL, USA) with a two-tailed significance level of $\alpha = 0.05$. To investigate the relationship between longevity and fecundity, Pearson Correlation and Scatter plot analyses were performed.

2.3 Results

2.3.1 Study population in young and old NZ sheep breeds

A total of 1826 blood samples from 40 flocks were collected for the longevity study. These comprised 1018 older ewes (from 3 to 16 years of age) and 808 younger ewes (two-tooth replacement ewes).

The data shown in table 2.1 compares the mean age in the younger and older groups. The average age of the ewes was 5.35 years (ranged from 2 to 16 years). While the young population had all 2 years old, the old population had age ranges from 3 to 16 years. The oldest sheep was 16 years old in this study.

In table 2.2, the number of sheep of each age in years and the proportion of the total is reported. The majority of sheep in the older group were between 7 and 9 years old.

Table 2.2. Young and old age groups of sheep used in this study.

Category	Age	Number (n)	Percentage (%)
Young	2	808	44.2
Old	3	1	0.1
Mean	4	26	1.4
(8.1 years)	5	62	3.4
	6	103	5.6
	7	175	9.6
	8	288	15.8
	9	181	9.9
	10	105	5.7
	11	57	3.1
	12	17	0.9
	13	1	0.1
	14	1	0.1
	16	1	0.1
Total		1826	100

2.3.2 Average number of lambs raised for the different age groups

Six hundred and ninety eight of the older ewes had records of their lambing performance over their life-time made available. An average per annum lambing performance was calculated as an indication of fecundity. This ranged from an average 1.40 to 2.27 lambs per year, with a mean of 1.79 lambs per year.

Table 2.3 shows that the average number of lambs raised per ewe, per year, decreased gradually with increasing age.

Table 2.3. Average number of lambs rasied for different age groups.

Age group (Year)	n	Percentage (%)	Average lambs raised/year
3-4	24	3.4	2.27
5	58	8.3	1.95
6	63	9.1	2.01
7	148	21.2	1.79
8	156	22.3	1.76
9	127	18.2	1.62
10	65	9.3	1.73
11	42	6.1	1.76
12	12	1.7	1.66
13-16	3	0.4	1.40
Total	698	100	1.79

2.3.3 Average number of lambs raised for the different NZ sheep breeds

The main breeds selected in this study were the Coopworth, Corriedale, NZ Romney and Merino. The NZ Romney is the most common breed in New Zealand and makes up about 41% of the national flock in 2007 (Meat & Wool New Zealand, 2007), but in this study Coopworth was the dominant breed, as the research was supported by the Coopworth Society of New Zealand.

Table 2.4 shows the averages of age and number of lambs raised in the different breeds. Among the breeds, the average lambing rate per year was highest in the Texel breed and

lowest in the Merino. However, this table was not analysed for further tends as it is unlikely that the selected sheep were representative of the whole breed and hence, these average cannot be interpreted as being breed-specific or typical.

Table 2.4. Average number of lambs raised for different breeds.

Breed	Number of farms	n	Average lambs raised per ewe per year	Average ewe age
Coopworth	9	439	2.11 ± 0.03	6.4 ± 0.10
Romney	8	340	1.91 ± 0.03	8.6 ± 0.11
Merino	8	326	1.28 ± 0.03	8.7 ± 0.12
Corriedale	8	318	1.65 ± 0.04	8.4 ± 0.12
Polwarth	2	129	1.65 ± 0.05	7.9 ± 0.19
Texel	2	60	2.32 ± 0.08	7.1 ± 0.23
Hampshire	2	40	2.06 ± 0.08	5.6 ± 0.33
Kelso	1	174	NA	8.6 ± 0.11
Total	40	1826		

2.3.4 The relationship between longevity and fecundity

Data from the ages of sheep and the average lamb per year were used to explore the relationship between longevity and fecundity.

A weak negative Pearson correlation between longevity and fecundity (measured as number of lambs raised) was observed ($r = -0.248$, $P < 0.001$).

The correlation between longevity and fecundity traits is illustrated in Figure 2.1. Age only accounts for 6% ($R^2 = 0.059$) of the variation in fecundity. This suggests that although older ewes do tend to have fewer lambs, other characteristics may be better predictors of fecundity than age, at least over the range of ages studied.

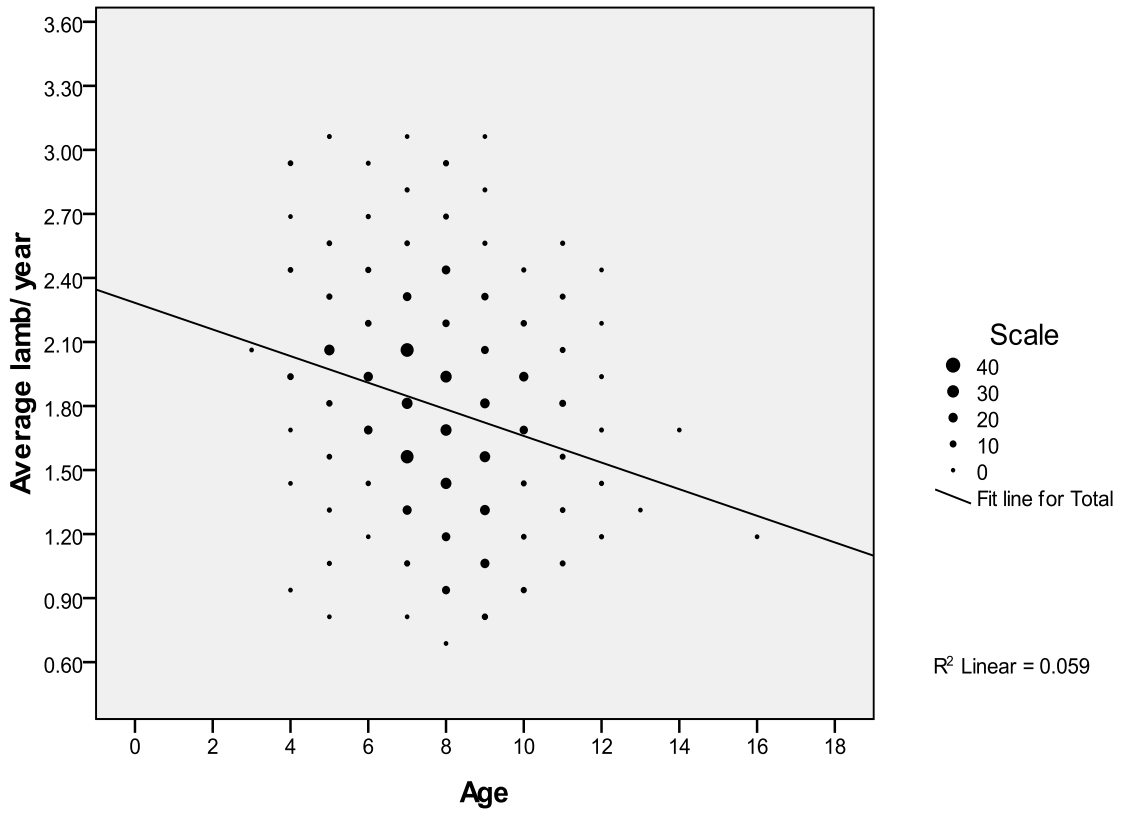


Figure 2.1. A correlation between longevity and fecundity in sheep.

2.4 Discussion

The effect of aging on survival and reproductive success are key elements of the trade-off theory, which suggests that reproductive costs could affect longevity in mammals. This is also known as reproductive senescence.

In the sheep industry, profitability in part depends on the number of lambs born for meat production per ewe, and over that ewe's life-time. Longer-lived ewes, provided they remain reproductively active throughout their lives, are effectively more efficient to farm as fewer replacements are needed. However, in studying sheep longevity it is necessary to investigate whether increasing their life-span is associated with a decrease in fertility as would be suggested by the trade-off theory.

Reproductive senescence has been shown in both short-lived mammals (Hoogland, 1995; Wauters & Dhondt, 1989) and long-lived mammals (Green, 1990). In many long-lived mammals and birds, longevity and reproduction patterns tend to follow a bell-shaped curve

(Jorgenson et al., 1997; Newton & Rothery, 1997), whereby reproductive ability increases after sexual maturity, becomes stabilized and then at some stage decreases until death. Reproductive senescence in mammals does not usually begin at sexual maturity or with the first reproduction (Gaillard et al., 1994), but at some time after these events.

In livestock, most measures of reproductive performance (including litter size, birth weight, and neonatal survival) usually remain approximately constant, only declining near the end of the animal's life-span. However, the life-span of livestock often depends on the ability to avoid culling (Ducrocq et al., 1988) and natural longevity is therefore masked behind a management practice that prematurely abbreviates life-span.

A recent study has investigated reproductive senescence in bighorn sheep (*Ovis canadensis*) (Festa–Bianchet & King, 2007). According to this research, reproductive senescence in bighorn sheep begins 6-7 years after the onset of survival senescence. The study confirmed the existence of reproductive senescence, but established no negative relationship between early and late reproductive success, or between early reproductive success and longevity. The study also confirmed that only ewes of higher phenotypic quality survived long enough to reach reproductive senescence. This suggests that bighorn ewe longevity had a positive influence on life-time reproductive success. Whether this is the case in domestic sheep (*Ovis aries*) is unknown.

In this thesis, the level of reproductive senescence was very similar to that reported for other female ungulates (Loison et al., 1999) including the bighorn sheep (Festa–Bianchet & King, 2007), with declining at the end of the ewes life. However, the relationship between longevity and fecundity in these NZ sheep was weak and negative. This suggests that although older ewes do tend to have fewer lambs, other factors such as farm management practices and availability of quality nutrition may be better predictors of fecundity, than age.

The investigation of the relationship between longevity and fecundity revealed in this study may have been affected by a number of things. Firstly, farmers were asked for number of lambs that survived to weaning, but they may have accidentally included lambs that were born, but died at birth, or between birth and weaning. This would affect the assessment of ewe fecundity and also possibly any decision to cull them.

For lamb survival, the first 48 hours after birth are critical time (Kerslake et al., 2005). Around 70% of lamb mortality occurs between birth and weaning and lamb mortality is generally associated with a lower birth weight (Brien et al., 2010; Scales et al., 1986).

Although ewes can be highly fecund, producing multiples lambs (twins and triplets), the multiple lambs have a lower average birth weight and are therefore more likely die between birth and weaning compared to single-born lambs (Everett-Hincks & Dodds, 2008; Kerslake et al., 2005). Being excessively fecund may therefore pose a productive cost.

Secondly, ewe culling decisions are based on production performance (wool and meat production and reproductive performance), animal health (structural unsoundness and temperament), management considerations and varying market opportunities. As ewe culling criteria may vary widely from farm to farm and season to season, the decision to replace ewes may reflect things other than just their reproductive performance, and this might affect their ability to realise their full reproductive potential.

This thesis has revealed that longevity and fecundity appeared to vary among the eight breeds studied. The Texel had the highest fecundity and Merino had the lowest. Different sheep breeds are characterised by different physical, productive and reproductive features. In this thesis, eight breeds were investigated from 40 different flocks. These flocks were subject to different management regimes, hence differences between flocks may affect either variation in longevity and fecundity within a breed or variation in flock management. In addition, the Coopworth and Romney breeds are dual purpose and suited for meat and wool production. Selection for two separate production systems in these breeds may also affect both their fecundity and longevity. For example, when these sheep are selected primarily for meat production, reproductive performance is the most important factor contributing to the efficiency of meat production, but this is less important for wool as the value is derived primarily from the ewe and not how many lambs she has.

An understanding of the factors governing reproductive success has fundamental implications for this study. From a trade-off point of view, artificial selection for production traits should theoretically lead to a deterioration of longevity. This has certainly been demonstrated in dairy cattle where selection for reproductive success and production traits including increased milk yield, reduced live weight and increased growth rate has resulted in negative correlated responses in fitness or longevity (Essl, 1998; Wall et al., 2006). On the other hand, studies of undomesticated species of sheep suggest that individuals with high life-time reproductive performance have greater longevity (Clutton-Brock et al., 1996; Hamel et al., 2009). Although a correlation between longevity and fertility/fecundity has been reported for many species, few studies have explored this in domestic sheep. In this thesis, there was a

negative correlation between longevity and fecundity, but it was weak, suggesting that selection for increased longevity is unlikely to adversely affect ewe fecundity.

In conclusion, reproductive success remains fairly steady until the end of the life-span, and some ewes remain reproductive beyond 12 years of age. Therefore, it would seem plausible to be able to select for long-lived ewe without having a major effect on their ability to produce lambs. The long-lived ewes would bring more reproductive opportunities, that are lost if they need to be regularly replaced. However, if ewes are kept longer, then there may be other costs to productively, including a reduced potential for genetic gain as generation time would increase.

Chapter 3
An association between longevity and variation in *IGF1R*

IGF1 signalling is considered to be a key determinant of somatic growth and adult body size (Rogol et al., 2002). Signalling driven by the binding of IGF1 to the IGF1 receptor (IGF1R) is a principle mechanism underpinning both metabolism and growth in animals, with the receptor being widely expressed across many cell types in both foetal and postnatal tissues.

▪ A generalised role for IGF1 in growth and metabolism

IGF1 is a small peptide (molecular weight 7,647) with a high sequence and structural homology to insulin (Kim & Accili, 2002; Laron, 2004). It is an anabolic hormone and found in almost every cell in the body, including muscle, cartilage, bone, liver, kidney, nerve, skin, and lung cells.

IGF1 is part of a complex metabolic system, with both GH-like and insulin-like effects (Laron, 2004). In a primary role, it affects somatic growth and differentiation by inducing a variety of growth factors, while in a secondary role it operates through insulin-like mechanisms. The latter play an important role in glucose metabolism by increasing insulin sensitivity and lowering glucose levels. The activity of IGF1 at the cellular level is largely mediated by IGF1R, although IGF1 can also bind to the insulin receptor (IR), with a low affinity.

Early observations revealed that in growing animals IGF levels increase with post-natal age to puberty (Imran et al., 2010). Serum concentrations in neonatal animals are relatively low, but gradually increased during maturation. They then gradually decline through adulthood. Excess IGF1, or a deficiency of it, can cause abnormal growth and other metabolic disorders. For example, in humans, abnormally high IGF1 levels have been linked to breast cancer, prostate cancer and certain types of colon cancer (Ouban et al., 2003).

IGF1 has been shown to mediate many growth-promoting effects (Guevara-Aguirre et al., 1997; J. L. Walker et al., 1992) in experimental animal systems. These effects were assessed using different doses and different routes of administration of IGF1 (Mauras & Haymond, 1996). If a low dose of IGF1 is used, it stimulates protein anabolic activity by selectively increasing whole body protein synthesis without affecting the rate of proteolysis,

whereas in higher doses IGF1 suppresses proteolysis (Mauras & Beaufrere, 1995; Turkalj et al., 1992).

The growth-promoting effects of IGF1 have been observed in many tissues, including bone (J. I. E. Wang et al., 1999), cartilage (Jenniskens et al., 2006), adipose cells (Wright & Hausman, 1995) and muscle (Barton-Davis et al., 1998) (Figure 3.2). In bone and cartilage, IGF1 promotes the growth of long bones at the epiphyseal plates, where there are actively proliferating cartilage cells (Jenniskens et al., 2006). This 'growth support' ceases once the epiphyses of the long bones fuse at the end of adolescence. Excessive GH after this time leads to progressively dysmorphic growth called acromegaly (a chronic metabolic disorder).

IGF1 also mediates bone turnover and bone mineral density. In adipose cells, IGF1 stimulates lipid synthesis and reduces lipolysis in an opposite fashion to GH (Wright & Hausman, 1995). In muscle cells, it increases the number of cells (hyperplasia) and the growth of new muscle fibre (mitogenesis), as well as inducing skeletal muscle hypertrophy. IGF1 is stimulated by nutrient intake through a complex interaction with GH. GH provides a mechanism for surviving periods of food deprivation, and hence its major function is catabolism. GH increases lipolysis to produce energy substrates and inhibits hepatic gluconeogenesis (Gasco et al., 2010). These effects increase blood glucose levels and reduce the need for dietary sources of carbohydrate.

IGF1 also has anabolic effects on the body. It stimulates peripheral tissue uptake of glucose, which lowers blood glucose levels (Berneis & Keller, 1996; Mauras & Haymond, 2005). Thus, during starvation, circulating IGF1 decreases rapidly. Furthermore, it is a major inhibitor of GH secretion, via a negative feedback control system (Thakore & Dinan, 1994), and this plays a role in maintaining metabolic balance by controlling whether metabolism will be anabolic or catabolic.

Circulating (endocrine) IGF plays a major role in glucose homeostasis compared to locally secreted (paracrine/autocrine/free) IGF1 (Phillips et al., 1990). Several authors have demonstrated the link between IGF1 and glucose homeostasis (J. L. Liu et al., 2000; Ohlsson et al., 2000; Sjögren et al., 2001). Liu et al. (2000) generated a mouse that specifically lacked IGF1 expression in the liver (Liver-specific IGF1 deficient: LID), the primary site of endocrine IGF1 production. While mice without the IGF1 gene will not generally survive, LID mice survive with normal growth and no obvious malformation. This suggests that GH can stimulate IGF1 production in extra-hepatic tissues (paracrine/autocrine production) and

that this is enough to produce body growth. In these LID mice circulating and serum levels of IGF1 were decreased by approximately 80%. Liu et al. (2000) also demonstrated the mice had specifically impaired "insulin sensitivity" in muscle and compensated for their low total IGF1 levels with hyperinsulinemia. This hyperinsulinemia was an attempt to maintain normoglycemia, as IGF1 administration reduced insulin levels and increased peripheral insulin sensitivity. This result suggests a direct role for circulating IGF1 in modulating insulin sensitivity and irrespective of free IGF1 levels. Overall, liver derived IGF1 has been confirmed as an important factor in maintaining normal carbohydrate metabolism, while extra-hepatic IGF1 is important in normal growth.

The control of cell growth and metabolism is critically important to survival. IGF1 regulates growth during the prenatal period as well as survival during adulthood. For example, caloric restricted mice have increased longevity and decreased IGF1 levels without an apparent effect on body size (Bartke et al., 2008). Indeed, when food is scarce, adult animals typically enter into a semidormant state allowing them to survive periods of food deprivation. Studies of GH/IGF1 deficient mice (Ames dwarf and GHRKO (growth hormone knockout)) have also revealed that they have increased longevity (Al-Regaiey et al., 2005; Bartke et al., 2008). These results support the idea that the IGF1 signalling pathway and its control of metabolic activity are likely to be a general regulator of the rate of aging and primarily through the control of metabolism and growth.

- **Knockout mouse models (IGFs and IGF1R)**

The central role of the IGF axis in regulation of pre- and post-natal growth has been confirmed in gene-knockout mouse models (Baker et al., 1993; J. P. Liu et al., 1993). The GH and GH receptor gene-knockouts have normal birth weight, confirming that GH is not an important regulator of pre-natal growth. However, elimination of IGF1 and IGF1R by homologous gene recombination has demonstrated that IGF1 is important for pre-natal tissue development and growth. IGF1 gene-knockout mice had a birth weight of 60% of normal and subsequently severe postnatal growth failure (Baker et al., 1993; J. P. Liu et al., 1993).

IGF2 null mice are equally growth retarded at birth (60% of normal size), but grow normally in the postnatal period, suggesting that IGF2 is primarily responsible for pre-natal growth (Baker et al., 1993; J. P. Liu et al., 1993). The synergistic effect of IGF1 and IGF2 on pre-natal growth has been confirmed by the observation that mice with deleted IGF1 and IGF2 genes have a birth weight of only 30% of normal.

While IGF1R is important in mediating both IGF1's and IGF2's actions, IGF1R knockout mice are affected more profoundly and die of postnatal respiratory failure due to poor development of the respiratory muscles (J. P. Liu et al., 1993). This suggests IGF1R is pivotal in IGF1 and IGF2 mediated activity.

IGF2R is also called "the mannose-6 phosphate receptor" and binds IGF2 alone. The receptor inactivates IGF2 and makes less IGF2 available for binding with IGF1R (Taguchi & White, 2008). This function of IGF2R is well supported by knockout mouse models, as mice lacking functional IGF2R (KO-IGF2R) are overgrown by up to 30% relative to their wild-type littermates (Ludwig et al., 1996). Collectively these studies demonstrate that the IGF axis is critical for regulating body growth and tissue development, and is therefore also of potential importance in determining life-span.

- **The structure of the IGF1 receptor**

The human complementary DNA (cDNA) of *IGF1R* was first cloned and sequenced in 1986 (Yamashita & Melmed, 1986). It consisted of 4989 nucleotides and encoded a 1367-animo acid precursor. The dimeric IGF1R protein contains two extracellular α subunits (~135 kDa each) and two transmembrane spanning β subunits (95 kDa each), which are joined by disulfide bridges to form a heterotetrameric receptor complex (a β-α-α-β arrangement, Figure 3.1). The α and β subunits are derived from different sub-regions of the IGF1R cDNA.

Mammalian *IGF1R* genes typically contain between 19 and 22 exons, 11 that produce the α subunit and 9 to 11 that produce the β subunit. These exons span approximately 100 kb of genomic DNA.

The α subunit can be subdivided into six distinct protein domains as follows: an N-terminal receptor L domain (L1), a cysteine-rich repeat (CRR) domain, a second receptor L domain (L2), and three type III fibronectin domains. The β subunit contains a tyrosine kinase catalytic domain (Adams et al., 2000).

The first three α domains (L1-cys-rich-L2) of the IGF1R are important for IGF1 binding. Of these, the cysteine-rich domain of IGF1R is critically important, as it has a high-affinity binding activity. Mutations or variation in the α subunits of *IGF1R* can affect IGF1 binding and this may have a significant effect on the physiology of the IGF1 axis.

Following ligand (IGF) binding to IGF1R, its tyrosine kinase activity is activated, and this stimulates signalling through intracellular networks that regulate various physiological mechanisms including cell proliferation and cell survival.

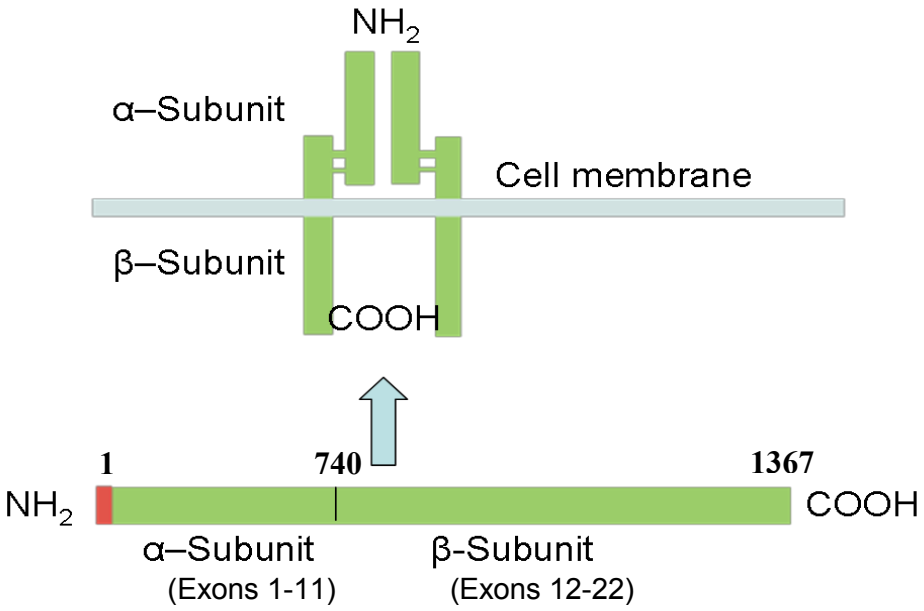


Figure 3.1. The structure of IGF1R.

- **IGF1R signalling and cellular metabolism**

IGF1R binds both IGF1 and IGF2 with high affinity and the insulin receptor (IR) binds insulin with high affinity. However, since both receptors share a high degree of structural and functional homology, IGF1R can also bind insulin and conversely the IR can bind IGF1 with a low affinity (LeRoith & Yakar, 2007).

The insulin-like growth factor binding protein (IGFBP) family has at least six members, which serve both as transporter proteins and as storage pools for IGF1 and IGF2. They control IGF1R action by regulating the bioavailability of IGF1 and IGF2 (Blakesley et al., 1996). Approximately 98% of IGF1 is bound to one of the six IGFBPs. Of these proteins, IGFBP-3 is present at the highest level in adults and is responsible for carrying IGFs to target tissues and prolonging it's half-life in circulation. However, in general, IGFBPs inhibit IGF activity by making them unable to bind to IGF1R and IGF2R. All six IGFBPs have been shown to inhibit IGF1 and IGF2 activities, but IGFBP-1, -3, and -5 also stimulate the action of IGF1 and IGF2 (Firth & Baxter, 2002).

Once the IGFs are able to bind their receptors, IGF1R can initiate signalling cascades via its cytoplasmic domains (Figure 3.2)

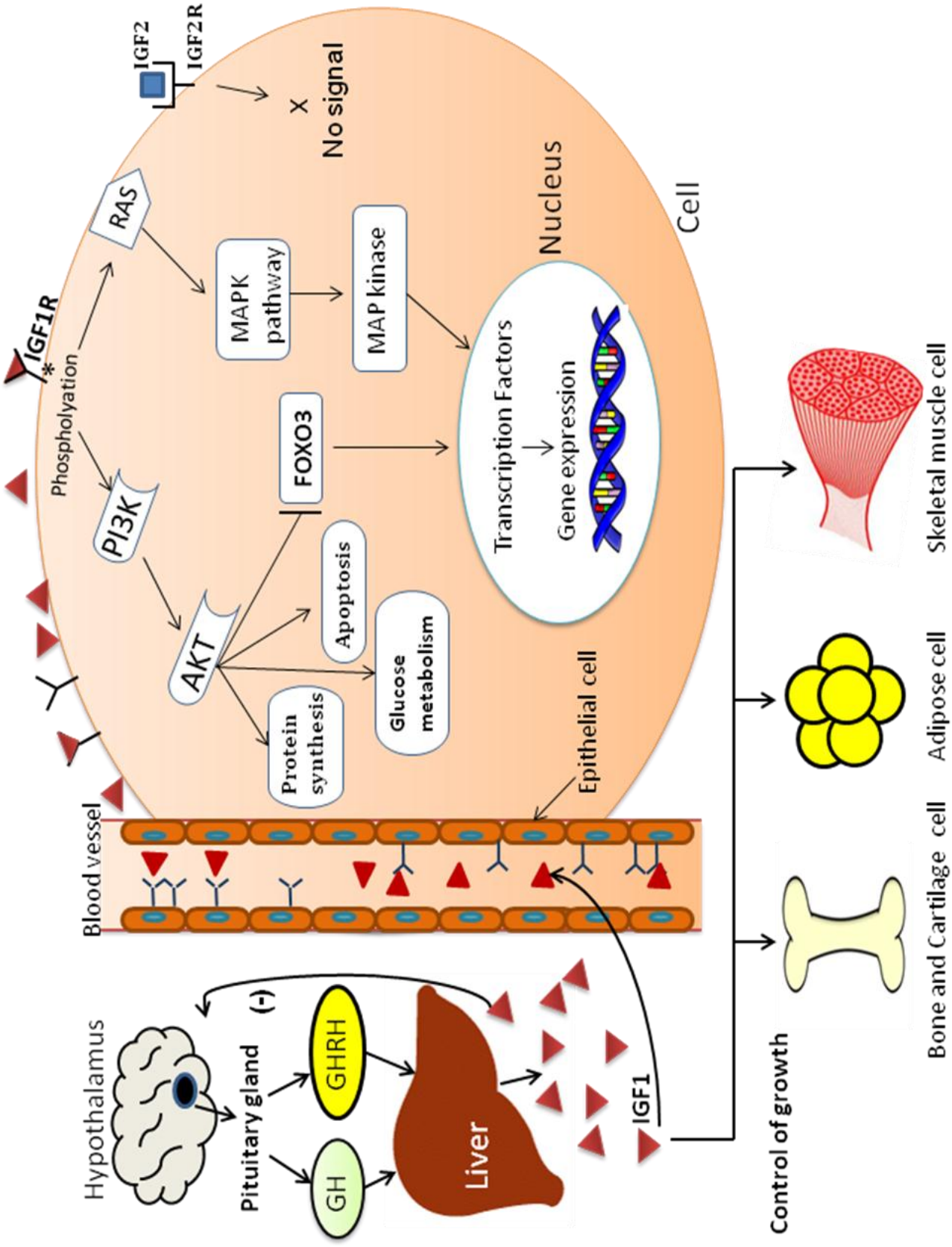


Figure 3.2. The physiology of GH and IGFs.

Downstream IGF1R signalling involves autophosphorylation and subsequent tyrosine-mediated phosphorylation of the Src-homology collagen protein (Shc) and insulin receptor substrate (IRS) proteins -1, -2, -3, and -4. The IRSs serves as docking proteins and can activate two well-characterized IGF1 signalling pathways; the MAPK pathway and the PI3K pathway (Figure 3.3). Activation of these signalling pathways induces different biological activities, including metabolism, cell growth, differentiation, migration and survival (Tatar et al., 2003).

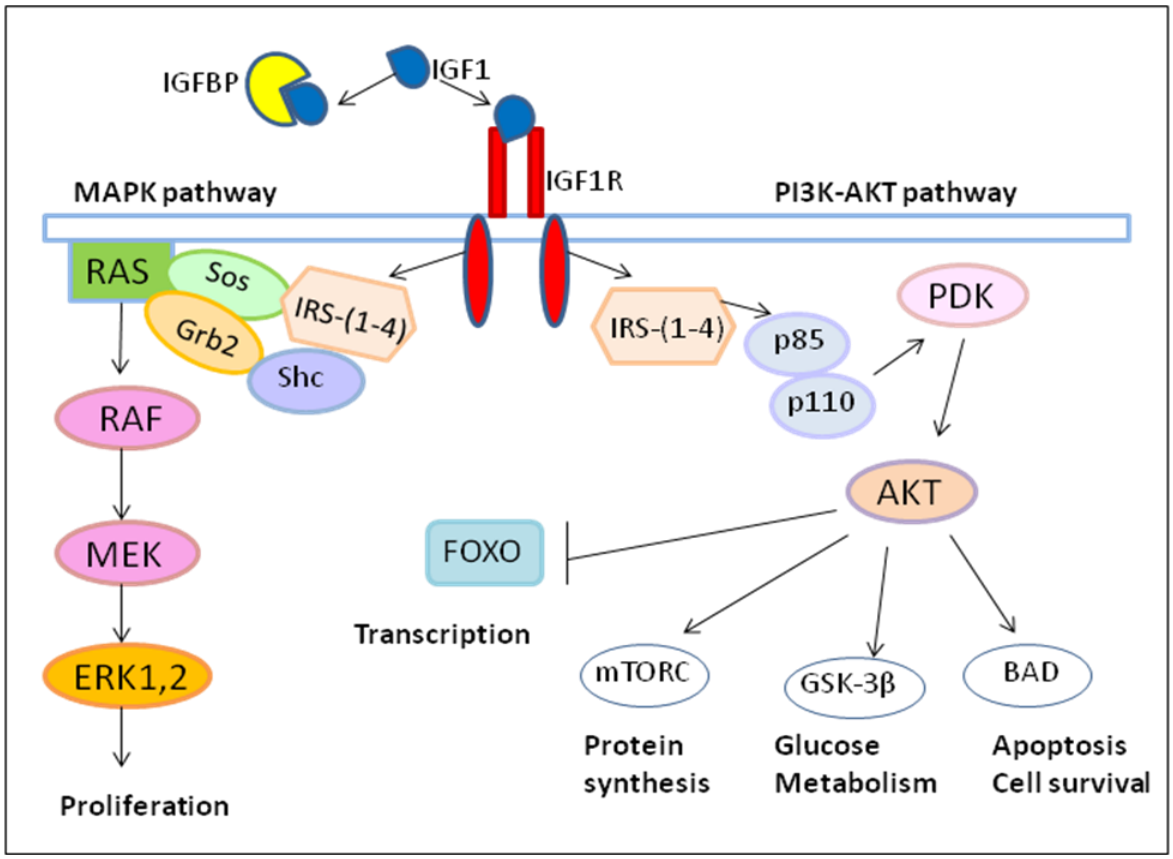


Figure 3.3. IGF1R activation and downstream signalling. Following ligand (IGFs) binding to IGF1R, tyrosine kinase activity of IGF1R is activated, and this stimulates downstream networks include the Ras/Raf/MEK/ERK and the PI3K-AKT pathways. The MAPK pathway is involved in cell survival, differentiation, proliferation, and protection against apoptosis. The PI3K-AKT pathway is involved in protein translation, cell survival, proliferation, apoptosis, and glucose metabolism

In the mitogen-activated protein kinase (MAPK) pathway, the proximal substrates for IGF1R include insulin receptor substrate (IRS) proteins 1 to 4 and the Src-homology collagen protein (Shc) (Firth & Baxter, 2002). Following activation of IGF1R, Shc and the IRSs bind to a complex consisting of growth factor receptor bound-2 (Grb2) and Son of sevenless (Sos).

This complex allows the activation of Ras and its downstream cascade, the Ras/Raf/MEK (MAPKK: mitogen activated protein kinase kinase) pathway.

Ras is a small guanosine triphosphate protein which is anchored to the inner surface of the plasma membrane (Figure 3.3). It is able to interact physically with several alternative downstream signalling partners. The first of the Ras effectors is the Raf kinase. After activation of Raf by Ras, Raf becomes phosphorylated, acquires active signalling capability and proceeds to phosphorylate and activate a second kinase known as MEK. MEK can phosphorylate serine/threonine residues as well as tyrosine residues, which will phosphorylate two other kinases, the extracellular signal-regulated kinases 1 and 2, commonly referred to as ERK1 and ERK2 (Boulton et al., 1991; Robbins et al., 1992). Phosphorylated and activated, each ERK then regulates various cellular processes including cell proliferation, differentiation, and protection against apoptosis.

In the PI3K pathway, IRS is the first adapter protein that is activated by IGF1R (Figure 3.3). Activated IRS also interacts with the p85 regulatory subunit of PI3K, leading to activation of catalytic subunit p110 of PI3K. The activation of PI3K leads to an increase in phosphatidyl inositol 3,4,5 triphosphate (PIP3) levels that induces the recruitment of AKT (protein kinase B). This allows the constitutively activated 3'-phosphoinositide-dependent kinase (PDK)-1 and PDK-2 to phosphorylate and activate AKT.

The activation of AKT mediates various cellular processes, including increased protein synthesis and cell growth by inhibition of the mTORC1 complex (mammalian target of rapamycin complex 1) (Datta et al., 1999), increased conversion of glucose to glycogen via inhibition of glycogen synthase kinase-3β (GSK-3β) activity (Asano et al., 2007) and increased proliferation and survival by activation or inhibition of anti-apoptotic factors such as BAD (pro-apoptotic protein of the Bcl-2 family), and nuclear factor kappa-B (Guertin & Sabatini, 2005) (Figure 3.3).

The mammalian Forkhead members of the class O (FOXO) transcription factor family are well-characterised substrates of AKT. Activated AKT in turn represses the FOXO transcription activity by direct phosphorylation. Phosphorylated FOXO remains inactive in the cytoplasm. In the absence of the AKT signal, FOXO becomes dephosphorylated and then transported into the nucleus, where it modulates the transcriptional activity of its target genes, which control among other things cell-cycle, cell death, cell metabolism and oxidative stress. Recent studies in nematodes (Lin et al., 1997) and insects (Hwangbo et al., 2004; Ogg et al.,

1997) have shown that having consistently activated FOXO as a consequence of insufficient IGF1 deficient signalling, can extend life-span. Accordingly, one of the main mechanistic outputs of IGF1 signalling is inhibition of the FOXO family.

- **The IGF1R gene and its homology across species**

Genes involved in the IGF1 pathway appear to be conserved from lower order species to complex organisms, reflecting the observation that they share a similar role across species ranging from *C. elegans* to humans (Barbieri et al., 2003; Xuan & Zhang, 2005).

Figure 3.4 is a phylogenetic tree constructed based on alignment of the full-length nucleotide sequence of IGF1R mRNA from various species. In the phylogenetic tree, species are clustered together according to similarity of their gene sequences. Table 3.1 was also derived from the same references as mentioned Figure 3.4. Humans, monkeys, cows, pigs, mice and rats; all mammals are very similar with over 95% nucleotide sequence identity (Table 3.1). This genetic relatedness of mammals allows us to speculate that the gene sequences will be similar in other mammalian species, including sheep. Therefore what we understand about the regulation of longevity in other species (e.g., humans, cows and mice) could be used to try and understand what is occurring in sheep. In effect, we expect those genes associated with variation in longevity in mice, humans and other characterised species, to also be associated with longevity in sheep.

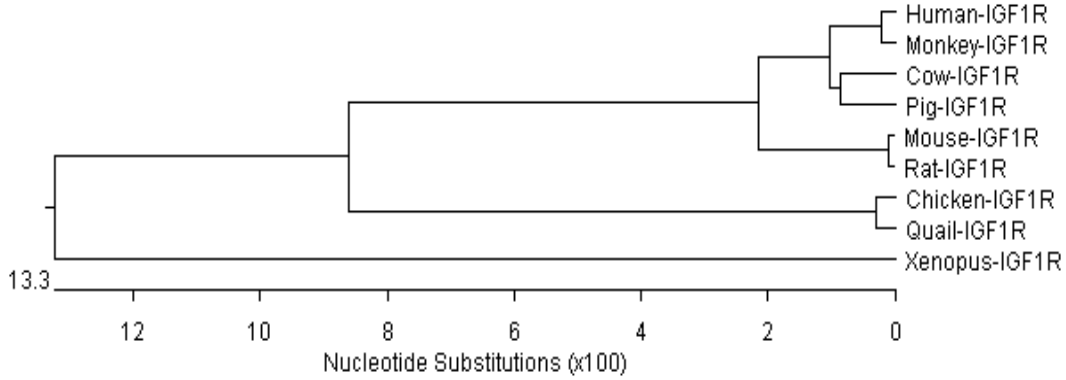


Figure 3.4. Phylogenetic tree of *IGF1R* sequences from various species. The phylogenetic tree was constructed by the neighbour-joining method, using the multiple alignment algorithms in the MegAlign package (DNAStar Inc., Madison, WI, USA). The full-length nucleotide sequences of IGF1R mRNA were extracted from GenBank. The GenBank database accession numbers are as follows AAI13611.1 for Human (*Homo sapiens*);

XP_001100407 for Monkey (*Macaca mulatta)*; DAA17722.1 for Cow (*Bos taurus*); NP999337.1 for Pig (*Sus scrofa*); NP034643.2 for Mouse (*Mus musculus*); NP434694.1 for Rat (*Rattus norvegicus*); NP990363.1 for Chicken (*Gallus gallus*); BAF73401 for Quail (*Coturnix japonica*); AF055080.1 for Xenopus (*Xenopus lavevis*)

Table 3.1. Amino acid sequence homology of IGF1R from various species.

(%) Identity	*Human*	*Monkey*	*Pig*	*Cow*	*Mouse*	*Rat*
Human	100					
Monkey	99.6	100				
Pig	98.4	98.5	100			
cow	97.6	97.6	98.3	100		
Mouse	95.7	95.8	95.5	94.7	100	
Rat	95.3	95.4	95.2	94.4	98.9	100

- **IGF1R and longevity**

Longevity is associated with reduced levels of IGF1 signalling in *C. elegans* (Hansen et al., 2007; Tullet et al., 2008), *D. melanogaster* (Giannakou & Partridge, 2007) and mouse models (Andrzej, 2006). It is hypothesised that with lower levels of IGF1 signalling, the metabolism of the organism shifts from growth and food intake to cellular maintenance and stress resistance. This hypothesis has been investigated in caloric restricted rodent models (Liang et al., 2003; Turturro et al., 1994). The caloric restricted rodents decrease the level of IGF1R signalling. Reduced IGF1 signalling stimulates starvation responses, which reduces plasma insulin levels and enhances insulin sensitivity as well as stress resistance. These patterns or changes are also seen in long-lived mammals (Masoro, 2005).

Studies have shown that complete or partial loss-of-function mutations in genes encoding components of the IGF1 signalling pathway result in extension of life-span in *C. elegans* (Kenyon, 2011), *D. melanogaster* (Clancy et al., 2001) and mice (Quarrie & Riabowol, 2004). In nematodes, DAF2 is similar to the insulin receptors and IGF1 receptors found in humans and other mammals. The nematode *C. elegans* lives for only a few weeks; but mutation in *daf-2* was found to double the life-span of the organism. In *D. melanogaster*, mutations in the insulin/IGF1 receptor extend female life-span by approximately 85%. In

mice, animals that lack one allele of the *IGF1R* gene show a 26% increase in mean life-span (Holzenberger et al., 2003). It therefore appears to be that the IGF1 signalling pathways role in longevity is evolutionarily conserved from nematodes to mammals and this suggests that altered signalling in this pathway may also influence life-span in humans and other mammals including sheep.

In humans, many studies have shown that genetic variation in *IGF1R* is associated with longevity, including studies in Italian cohorts (Bonafè et al., 2003), Ashkenazi Jews (Suh et al., 2008) and the Japanese (Kojima et al., 2004). In these studies, centenarians were more likely to have the longevity-associated variation in *IGF1R*.

Although genetic variations in *IGF1R* that is associated with longevity have been reported in a variety of mammals, there is little scientific literature about genetic variation in the receptor gene or its relationship with longevity in sheep. In this chapter, part of intron 2 and exon 3 of ovine *IGF1R*, were characterised to identify genetic variation and the association between this variation and longevity was tested.

3.1 Materials and Methods

3.1.1 Sheep investigated to define variation in *IGF1R*

To investigate genetic variation in ovine *IGF1R*, 100 New Zealand Romney sheep were studied in detail.

3.1.2 Sheep studied for longevity and fecundity analysis

Blood samples from sheep of known age and with lambing records were used (See chapter 2.2 for sheep studied and the collection of longevity and fecundity data) to assess the effect of genetic variation of *IGF1R* on sheep longevity and fecundity.

3.1.3 DNA extraction from blood on FTA cards

Blood samples were collected onto FTA cards (Whatman BioScience, Middlesex, U.K.), and genomic DNA was purified using the NaOH method described by H. Zhou (2006). In this method, a 1.2-mm blood disc was punched from each blood spot and was transferred into a 0.2 mL PCR tube. 200 µL of 20 mM NaOH solution was added to the tube and it was incubated for 30 min at room temperature. The solution was then discarded, and the disc was equilibrated in 200 µL of 1 × TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). After removal of the TE buffer, the disk was air-dried, prior to use in PCR.

3.1.4 PCR primer design and synthesis

Two PCR primers, 5'-CTCACACCCTGCCTGTC-3' and 5'-CACACTGACCTCTGGCTC -3', were designed to amplify intron 2-exon 3 of the ovine *IGF1R* gene. These were designed based on human and bovine *IGF1R* sequences (GenBank accession number AY332722, Ensembl transcript ID ENSBTAT00000028690, respectively) (Figure 3.5). The primers were synthesised by Integrated DNA Technologies (Coralville, IA, USA).

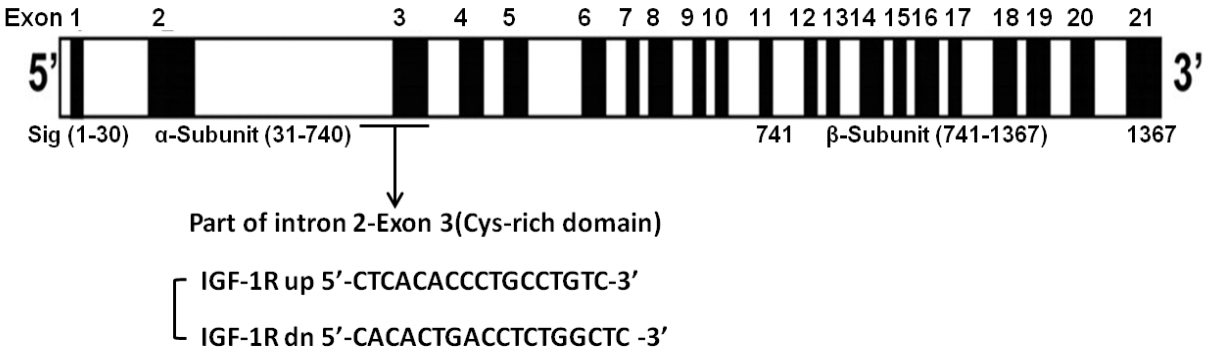


Figure 3.5. Location of the *IGF1R* primers used in this study.

3.1.5 Polymerase Chain Reaction-Single-Strand Conformational Polymorphism (PCR-SSCP) analysis

Amplification was performed in a 20 µL reaction containing the genomic DNA on one 1.2-mm punch of FTA paper, 0.25 µM of each primer, 150 µM of dNTPs (Eppendorf, Hamburg, Germany), 1.5 mM of Mg^{2+}, 0.5 U of Taq DNA polymerase and 1 × reaction buffer supplied. Amplification was carried out in an iCycler (Bio-Rad Laboratories, Hercules, CA, USA) and consisted of denaturation at 94 ºC for 2 min, followed by 35 cycles of 94 ºC for 30 s, 62 ºC for 30 s, and 72 ºC for 30 s, with a final extension step at 72 ºC for 5 min.

Amplicons were mixed with 7 µL of loading dye (98% formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene-cyanol), and after denaturation at 95 ºC for 5 min, samples were rapidly cooled on wet ice and then loaded on 16 cm × 18 cm, 12% acrylamide:bisacrylamide (37.5:1: Bio-Rad) gels with the addition of 3 % glycerol. Electrophoresis was performed using Protean II xi cells (Bio-Rad), at 280 V for 18 h at 28 ºC in 0.5 × TBE buffer. The gels were silver-stained according to the method of Sanguinetti et al. (1994). Genomic DNA representative of the unique SSCP patterns was amplified using *Pwo* SuperYield DNA polymerase (Roche Applied Science, Mannheim, Germany) and amplicons

were ligated into the pCR4Blunt-TOPO vector (Invitrogen, Carlsbad, CA). Only those plasmids for which the insert PCR-SSCP patterns matched with the genomic DNA template patterns were sequenced in both forward and reverse directions.

3.1.6 DNA sequencing and analysis of sequence diversity

Amplicons representing unique banding patterns were sequenced at Lincoln University, New Zealand. Sequence alignments, translations, phylogenetic tree, creation and comparisons were carried out using DNASTAR (Madison, WI, USA) and DNAMAN (Version 5.2.10, Lynnon BioSoft, Vaudreuil, Canada). The BLAST algorithm was used to search the NCBI GenBank (http://www.ncbi.nlm.nih.gov/) and Ensembl (http://www.ensembl.org) databases for homologous sequences of *IGF1R* in different species.

3.1.7 Genotyping of ovine *IGF1R* for longevity and fecundity trait analysis

A total of 1718 sheep were genotyped using the PCR-SSCP, conditions described above. These sheep were ewes of the NZ Romney (n = 340), Corriedale (n = 318), Merino (n = 322), Polwarth (n = 127), Kelso (n = 174) and Coopworth (n = 437) breeds from 36 flocks. Overall there were nine hundred and fifty one older ewes and seven hundred and sixty-seven young ewes (2 years old). Lambing information was available for six hundred and fifty-three of the older ewes for assessing their fecundity.

3.1.8 Statistical analyses

Data were analysed using SPSS version 17 (SPSS Science Inc., Chicago, IL, USA) at a significance level of $\alpha = 0.05$.

Unadjusted analyses exploring the relationship between *IGF1R* sequences and longevity and fecundity were undertaken using one-way analysis of variance. General linear mixed effects models (GLMMs) were used to assess the effect of the presence of each of the *IGF1R* sequences on longevity and fecundity. Sheep breed and flock were fitted as random factors in the model to accommodate variation from farm to farm in flock management, breeding ewe culling and replacement ewe selection. GLMMs were also used to explore the effects of the more common *IGF1R* genotypes (frequency $\geq$ 10%) on longevity and fecundity. Where significant or if tending towards significance these were further explored using pairwise comparisons (least significant difference).

Pearson *Chi*-square analysis was used to ascertain whether or not *IGF1R* genotype frequencies differed between older and younger ewes.

3.2 Results

3.2.1 Polymorphism of the ovine *IGF1R*

Amplicons of 346 bp were obtained with all the sheep DNA samples amplified. Upon SSCP analysis, three distinct banding patterns were detected (Figure 3.6). Either one or two of these patterns were observed for each sheep studied, which defines homozygous and heterozygous genotypes of ovine *IGF1R*, respectively.

Cloning of PCR amplicons representative of the unique SSCP patterns followed by DNA sequencing, revealed three different DNA sequences (Figure 3.7). All these sequences shared high homology to the reported human sequence (GenBank accession number AY332722) and sequences from other species, suggesting that they represent genuine ovine *IGF1R* sequences. The *IGF1R* sequences were named *A*, *B*, and *C* and deposited into the NCBI GenBank with the accession numbers EF669473-EF669475.

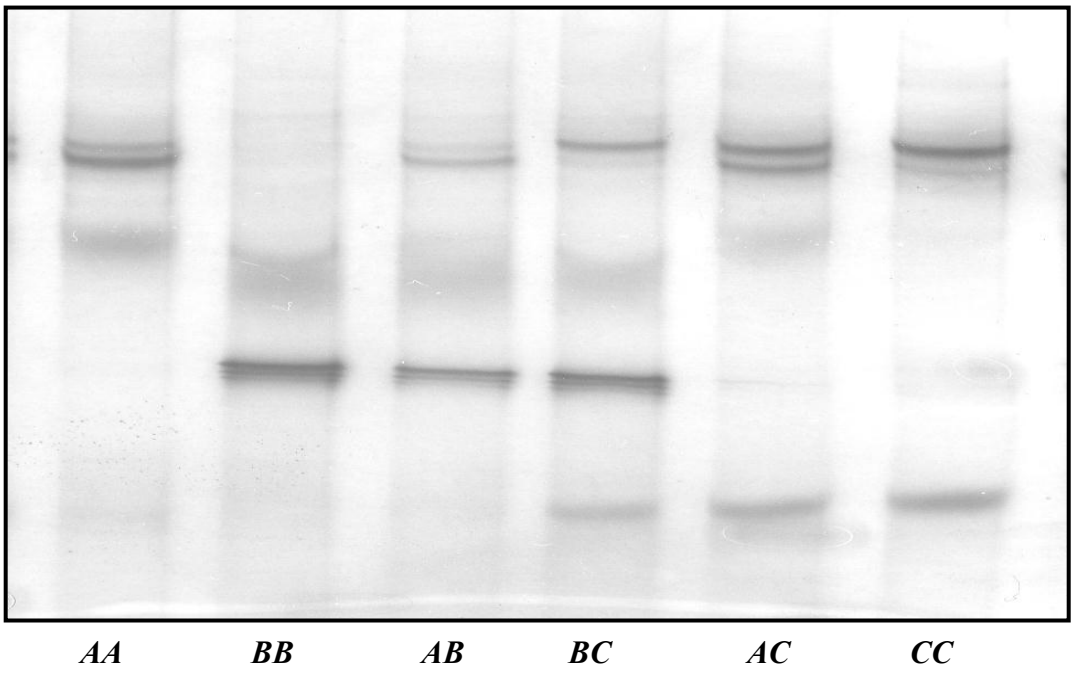


Figure 3.6. PCR-single-strand conformational polymorphism of the ovine *IGF1R*. Representative sheep for the three unique SSCP patterns, corresponding to genotype-combinations of three unique sequences (*A*, *B* and *C*) are shown

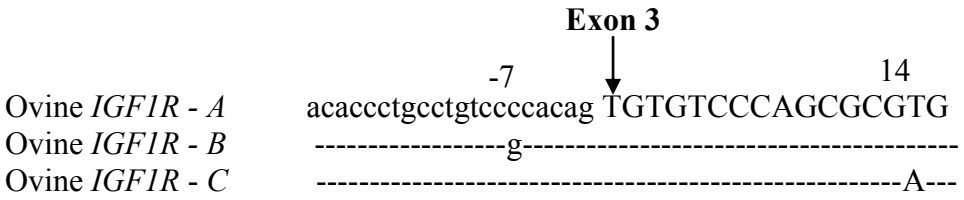


Figure 3.7. Sequence variation in a selected region of intron 2 and exon 3 of the ovine *IGF1R*. Bars represent nucleotides identical to the nucleotides of *A* sequence. Lower case and upper case letters refers to intron 2 and exon 3, respectively. The arrow shows the first nucleotide of exon 3 of ovine *IGF1R*, based on the human sequence (GenBank accession number AY332722)

3.2.2 An association between sheep longevity and variation in ovine *IGF1R*

Table 3.2 shows the genotype frequencies for the three sequences of *IGF1R*. The frequencies of each sequence in the sheep studied are shown in table 3.2. These frequencies were compared between the older ewes and the two year old replacement ewes. *Chi*-square test analysis suggested that there was a significant difference between them ($P = 0.009$).

Table 3.2. *IGF1R* genotype frequencies in older (3-16 years) and younger (2 years) ewe groups.

	Genotype						
	AA	*AB*	*AC*	*BB*	*BC*	*CC*	Total (n)
Older ewes	695	90	140	2	9	15	951
	72.5%	**9.4%**	**14.6%**	**0.2%**	**0.9%**	**1.6%**	
Younger ewes	597	78	73	6	4	9	767
	77.7%	**10.2%**	**9.5%**	**0.8%**	**0.5%**	**1.2%**	

In an analysis of all of the ewes (n = 1718), the *IGF1R C* sequence was associated with sheep age across breeds and flocks (Table 3.3). When correcting for breed, the presence of *IGF1R C* increased age by about half a year, with ewes that possess *IGF1R C* having a mean age of 6.08 ± 0.20, whereas ewes that did not possess the sequence had a mean age of 5.52 ± 0.09 ($P = 0.009$). When correcting for flock, there was also a significant increase in age, for the ewes carrying *IGF1R C* ($P = 0.024$). When the *C* sequence was present and absent, ewes had mean of ages of 5.52 ± 0.21 and 5.02 ± 0.08 years, respectively (Table 3.3).

To assess the effect of *IGF1R* genotype on longevity, only the common genotypes *AA, AB* and *AC* were included (Table 3.4) as genotypes *BB* (n = 8), *BC* (n = 13) and *CC* (n = 23) were rare. The GLMMs suggested an association, although not significant ($P = 0.064$) between age and genotype. A pairwise comparison revealed the difference to be between *AA* (5.0 ± 0.09 years) and *AC* (5.6 ± 0.22 years, $P = 0.019$) (Table 3.4).

The presence or absence of particular *IGF1R* sequences and *IGF1R* genotype GLMMs was not found to have any effect on the fecundity in the unadjusted or adjusted models (Table 3.5).

Table 3.3. Association of the ovine *IGF1R* variants with variation in longevity.

Sequence	status	n	Age (unadjusted means)	Breed adjusted	Flock adjusted
A	Present	1673	5.34 ± 0.12	5.61 ± 0.08	5.09 ± 0.08
	Absent	45	5.67 ± 0.70	5.41 ± 0.46	4.98 ± 0.46
			($P = 0.513$)	($P = 0.664$)	($P = 0.808$)
B	Present	189	5.30 ± 0.35	5.51 ± 0.23	5.60 ± 0.23
	Absent	1529	5.36 ± 0.13	5.62 ± 0.09	5.09 ± 0.08
			($P = 0.806$)	($P = 0.653$)	($P = 0.894$)
C	Present	250	6.23 ± 0.31	6.08 ± 0.20	5.52 ± 0.21
	Absent	1468	5.20 ± 0.13	5.52 ± 0.09	5.02 ± 0.08
			($P \leq 0.001$)	**($P = 0.009$)**	**($P = 0.024$)**

Table 3.4. Association of the ovine *IGF1R* genotype with variation in longevity.

Genotype	n	Unadjusted model	Flock adjusted model
AA	1292	5.2 ± 0.09^{a}	5.0 ± 0.09^{a}
AB	168	5.3 ± 0.25^{a}	5.1 ± 0.25^{ab}
AC	212	6.3 ± 0.22^{b}	5.6 ± 0.22^{b}
		($P \leq 0.001$)	($P = 0.064$)

[a,b] Different superscripts indicate the pair of means was significantly different ($p < 0.05$) in pairwise comparisons (least significant difference).

Table 3.5. Association of the ovine *IGF1R* sequences with variation in fecundity.

Sequence	status	n	Average lambs/year (Unadjusted means)	*P* values
A	Present	638	1.77 ± 0.19	Unadjusted *P* = 0.985
	Absent	15	1.77 ± 1.12	Breed adjusted *P* = 0.967
				Flock adjusted *P* = 0.835
B	Present	73	1.83 ± 0.56	Unadjusted *P* = 0.275
	Absent	580	1.76 ± 0.20	Breed adjusted *P* = 0.565
				Flock adjusted *P* = 0. 095
C	Present	103	1.83 ± 0.47	Unadjusted *P* = 0.157
	Absent	550	1.76 ± 0.21	Breed adjusted *P* = 0.125
				Flock adjusted *P* = 0.535

3.3 Discussion

Mammalian IGF1R is an evolutionarily conserved key regulator of the insulin-like signalling pathway and has been reported to influence metabolism and life-span in a variety of species (Barbieri et al., 2003; Richardson et al., 2004; Sepp-Lorenzino, 1998). However, this is the first report of an association between variation in this receptor and variation in life-span in sheep. This finding is consistent with reports in other species.

Three novel sequences (*A*, *B* and *C*) of *IGF1R* were identified in fragment of 346bp, which encodes part of intron 2 and exon 3 of this gene. The *A* and *B* sequences were differentiated by variation in intron 2 and the *C* sequence was identified by a synonymous substitution (bovine cDNA position at 211 G>A), which is located in exon 3.

In this study, the presence of the *C* sequence of ovine *IGF1R* was associated with ewe longevity. While this synonymous substitution does not result in an amino acid change, it may never-the-less be linked to other nucleotide changes in the coding regions, or to sequence variation elsewhere in the gene. This may affect gene expression and/or the function of IGF1R and hence affect sheep longevity.

Biochemical analyses and mutational studies have revealed that exon 3 of *IGF1R*, which encodes a cysteine rich region, is very sensitive to genetic mutation (Adams et al.,

2000; Denley et al., 2005). While we detected no changes in the likely amino acids derived from sequences *A*, *B* and *C*, the synonymous variation seen in *C* may affect mRNA processing or stability, thereby changing gene expression and accordingly the IGF1 signalling pathway. This would need to be investigated in a series of functional studies that focused on either IGF1R mRNA levels or surface expression of the receptor. Neither would be straightforward given the likely presence of the other chromosomal transcript of IGF1R. Breeding sheep for homozygosity of the *C* sequence would facilitate this investigation.

It is interesting to speculate why the variation reported here in *IGF1R* occurs and/or persists in the population of sheep studied. These sheep were selected for analysis and hence a population-based analysis of Hardy-Weinberg equilibrium would be inappropriate. However, the results suggest that despite *C* being more commonly found in older ewes, the *A* variant is proportionally more common in the younger ewe population. *A* is also the most common variant in the ewes studied, suggesting it may confer some advantage for ewes, especially younger ewes.

According to the "rate of living theory of aging" (Pearl, 1928), reduced metabolic rates in an animal should result in an increased life-span. That is, an inverse relationship exists between cellular metabolic rate and life-span. In mammals, the IGF1 signalling pathway has evolved to regulate metabolism and growth. Indeed, interventions such as dietary restriction (Masoro, 2005), gene mutation (Kenyon, 2001) and removal of the germ-line stem cells (Hsin & Kenyon, 1999), reduced the level of IGF1 signalling and are associated with increased life-span in model organisms. Reduced levels of IGF1 signalling have also been associated with stunted growth (dwarfism) and reduced adult body size in mammals (de Magalhães & Faragher, 2008). Therefore, it could be speculated that the *C* variant identified here is associated with lower growth rates in sheep, and the *A* variant is associated with higher growth rates. This would need to be confirmed by a separate study of growth rate in sheep, but this would be an anathema to sheep production.

In the genetic association study, longevity data was collected from different breeds that were derived from 36 farms. Few of the farmers had more than one breed and there is significant variation in longevity between farms presumably reflecting different management policies for retaining or culling ewes. These were commercial stud farms and while sheep die naturally, it is suspected that most sheep on these farms are culled by the farmers annually and/or on an ongoing basis for a variety of reasons including failure to become pregnant. Farm-related factors were a major determinant of longevity and hence "farm" needs to be

incorporated into any statistical analysis of longevity. Given that most farms only have one breed, then farm and breed are conflicted. Accordingly correcting for both factors in linear models may create a compounded error.

In this study both breed and farm were corrected for in adjusting longevity data. As a result, only the *C* sequence remained significantly associated ($P = 0.009$, $P = 0.024$, respectively) with longevity when breed and farm corrections were included. Given that the reason for culling sheep out of flocks are diverse and probably inter-related (e.g., combination of age, lack of fertility and health) it is nevertheless conceivable that factors like variation in fertility or resistance to disease may also explain the association we have found in this study.

In conclusion, the role of genetic variability at the IGF1R has been hypothesised to be evolutionarily conserved throughout the animal kingdom (Murphy, 2006). Therefore, the genetic variation found in ovine *IGF1R* in this study could be used as a potential way of improving longevity in livestock, but that is assuming the variation in this gene has no detrimental effects on sheep production.

Chapter 4
Identification of variation in ovine *FOXO3* and its association with longevity

- **The physiology of FOXO3**

In mammals, the transcription factor FOXO3 regulates hormonal, nutrient and stress responses and affects cell survival and metabolism. The ability to regulate FOXO3 activity is important to controlling these cellular functions and is dependent on several post-translational mechanisms, including phosphorylation, acetylation and ubiquitination (Boccitto & Kalb, 2011). These post-translational modifications alter FOXO3's subcellular localisation and affect its degradation, DNA-binding ability and transcriptional activity.

As a nuclear transcription factor, the primary function of FOXO3 is to bind to target DNA sequences as a monomer unit. DNA-binding studies have identified a consensus FOXO3 recognition element of sequence (G/C)(T/A)AA(C/T)AA (Biggs et al., 1999; Furuyama et al., 2000). Sequences that resemble this consensus have been identified in the promoters of many genes (Furuyama et al., 2000).

The best-defined FOXO3 targets include the cell-cycle inhibitors p27 and p21, growth arrest-and DNA damage-inducible gene 45 (GADD45), the stress response genes for manganese superoxide dismutase (MnSOD), the pro-apoptotic factor Bcl2-interacting mediator of cell death (Bim), the Fas ligand and glucose-6-phosphatase (G6Pase) (Greer & Brunet, 2005) (Figure 4.1). These target genes mediate various cellular processes including cell-cycle arrest, DNA repair, detoxification of reactive oxygen species (ROS), apoptosis, cell death and glucose metabolism. Taken together, these processes are crucial in enabling the cell to respond to environmental stimuli, including the availability of nutrients, changes in energy supply and other cellular stresses. Collectively, it could be concluded that FOXO3 either regulates cell survival or of very least is implicitly involved in it.

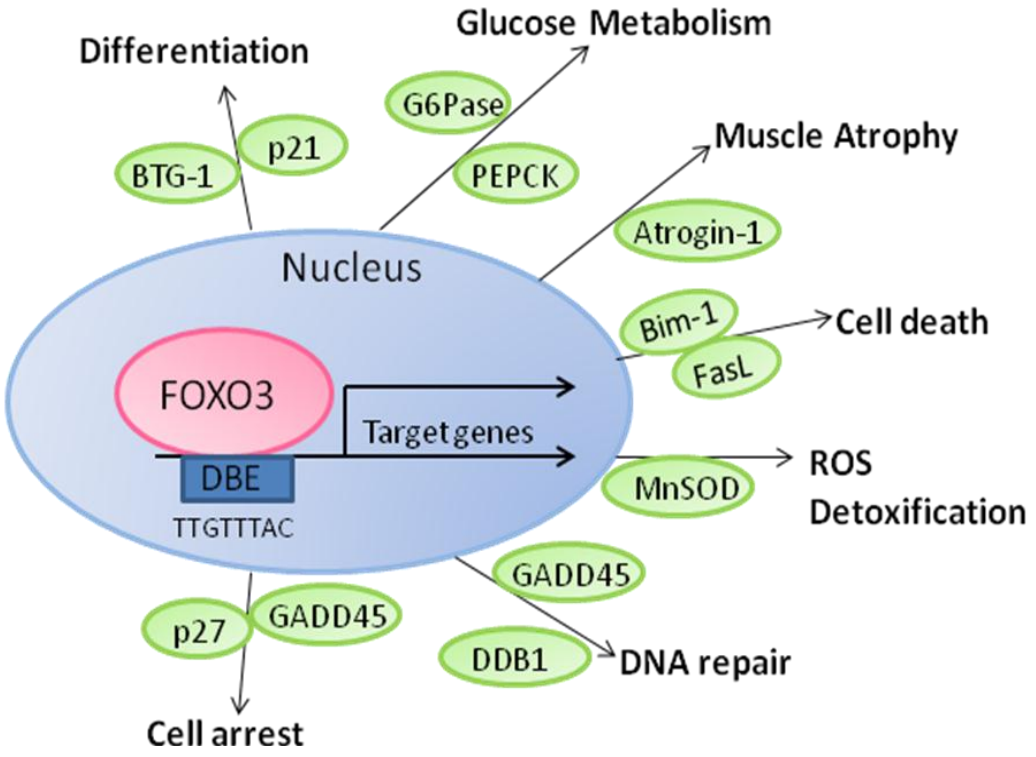


Figure 4.1. FOXO3 target genes and cellular roles. FOXO3 is located in the nucleus, where it induces the transcription of a variety of target genes. BTG-1, B-cell translocation gene 1; p21, cyclin-dependent kinase inhibitor 1A; p27, cyclin-dependent kinase inhibitor 1B; MnSOD, manganese superoxide dismutase; G6Pase, glucose-6-phosphatase; PEPCK, phosphoenolpyruvate carboxykinase; FasL, Fas ligand; GADD45, growth arrest and DNA damage-inducible protein 45; DDB1 damage-specific DNA-binding protein1; DBE, DAF-16 family member-binding element (adapted from Greer and Brunet, 2005)

FOXO3's ability to induce cell-cycle arrest and affect DNA repair and apoptosis, suggests an important role in regulating tumour suppression (Greer & Brunet, 2005). For example, a loss of FOXO3 function leads to a decreased ability to induce cell-cycle arrest, which leads to tumour development. The decreased ability to repair damaged DNA, due to the absence of FOXO3, could also lead to genomic instability. Abnormal cells that would normally undergo apoptosis may instead survive in the absence of FOXO3, and this would result in tumourigenesis.

FOXO3 "knockout-mouse" models have also been employed to understand the function of FOXO3 in tumourigenesis (Johnson et al., 2008). This study revealed that *FOXO3* null mice have over-proliferation of helper T cells, as well as Fas ligand-induced neutrophil apoptosis. The evidence also suggests that FOXO3 plays a tumour suppressor role in a variety of cancers (Greer & Brunet, 2005), and that this may promote organismal longevity.

In *C. elegans*, a homolog of FOXO3, called DAF-16, is a major mediator of defense against oxidative stress (Lehtinen et al., 2006; Lin et al., 1997). Study of longevity in *C. elegans* has shown that DAF-16 regulates the detoxification of ROS by up-regulating the expression of free radical scavenging enzymes, such as MnSOD (Honda & Honda, 1999). It has also been observed that activation of FOXO3 in cultured fibroblasts and neuronal cells results in resistance to oxidative stress (Kops et al., 2002). MnSOD is a major component of cellular defence mechanisms against the toxic effects of superoxide radicals.

Longevity is known to be associated with increased oxidative stress resistance in a variety of organisms (Beckman & Ames, 1998). The maintenance of highly regulated mechanisms to control ROS levels is essential for normal cellular homeostasis. ROS such as superoxide anions and hydrogen peroxide; the by-products of oxidative metabolism, are involved in many cellular signalling processes (Beckman & Ames, 1998). However, excess accumulation of ROS can result from defects in ROS scavenging and is believed to have an impact on cellular aging and the senescence process. Conversely, the ability to withstand oxidative stress has been correlated with enhanced longevity in several species (Beckman & Ames, 1998).

FOXO3 appears to play roles in controlling cell-cycle arrest, cell development and differentiation (Brenkman & Burgering, 2003). These roles are important in follicular development. A study by Castrillon et al. (2003) has demonstrated that FOXO3 is a crucial regulator of follicular development in mice. In this work, *FOXO3* null mice show a distinctive ovarian phenotype of global follicular activation leading to oocyte death, early depletion of functional ovarian follicles, and secondary infertility. These mice also displayed signs of premature aging. These symptoms resemble those seen in human premature ovarian failure (POF). This suggests that FOXO3 may regulate fertility in mammals as well as organismal longevity.

- **The regulation of FOXO3 activity**

FOXO3 activity is regulated at several levels and involves protein-protein interactions with cofactors, repressors and adaptors; and various post-translational modifications such as phosphorylation, acetylation, methylation and ubiquitination (Burgering, 2008; Calnan & Brunet, 2008; van der Vos & Coffer, 2008). Among these activation mechanisms, phosphorylation by the PI3K-AKT and AMPK pathways is known to tightly regulate FOXO3 transcriptional activity.

Phosphorylation by PI3K-AKT controls a shuttling system that modulates FOXO3 cellular localisation and this confines FOXO3 to either the nucleus or the cytoplasm (van der Heide et al., 2004) (see Figure 4.2).

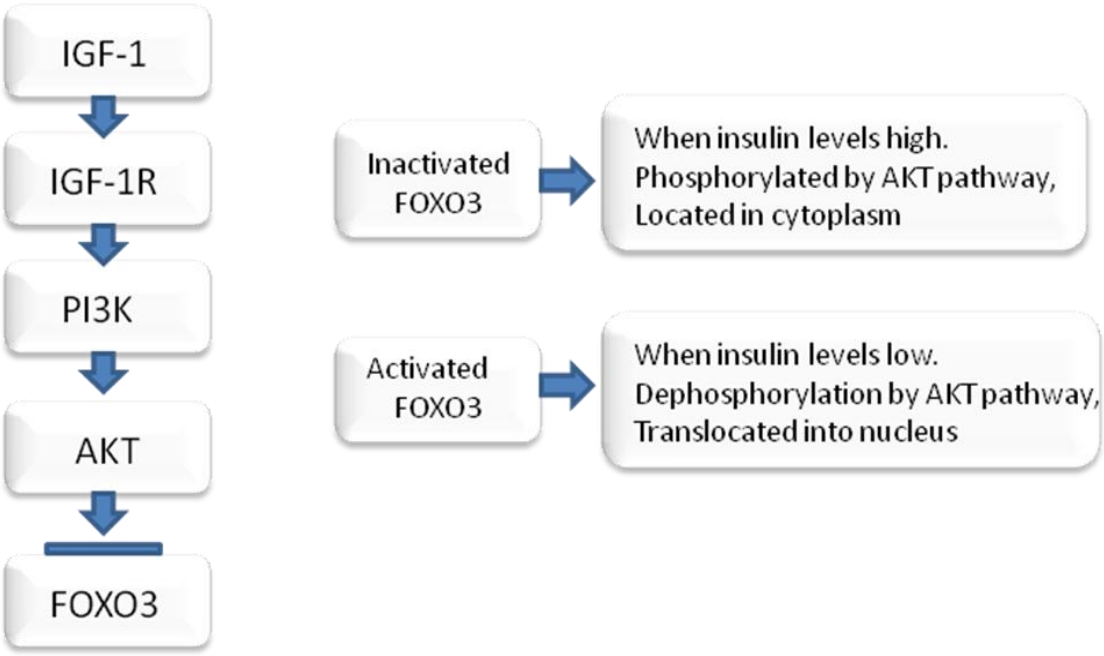


Figure 4.2. Regulation of FOXO3 by the IGF1 pathway. IGF1: insulin-like growth factor 1; IGF1R: IGF1 receptor; PI3K: phosphoinositide 3-kinase; PIP3: phosphatidylinositol (3,4,5)-trisphosphate; protein kinase B (PKB/AKT). FOXO3 mediates a transcriptional response to insulin signalling. Under conditions of abundant nutrients, FOXO3 is retained in an inactive state in the cytoplasm due to phosphorylation by AKT. When insulin levels fall, FOXO3 is dephosphorylated and translocated into the nucleus, where it stimulates transcription of other negative regulators of growth. In addition, activated FOXO3 increases expression of the insulin receptor gene, which may result in increased insulin sensitivity under low insulin conditions (adapted from Van Der Heide et al., 2004)

Regulation of FOXO3 requires activation of PI3K-AKT in response to cellular stimulation by the growth factors IGF, IGF2 and insulin. Binding of the growth factors or insulin to their receptors, triggers the recruitment and activation of PI3K, which in turn activates AKT. Upon stimulation with high levels of IGF1, IGF2 or insulin, three sites on the FOXO3 molecule (Thr32, Ser253, and Ser315 for human FOXO3, see also Figure 4.3 (c)) are phosphorylated by the AKT pathway to inactivate the protein. PI3K-AKT induced phosphorylation of FOXO3 generates binding sites for 14-3-3 proteins. Once phosphorylated, FOXO3 binds to these 14-3-3 proteins in the nucleus immediately before it relocalises to the

cytoplasm (Brownawell et al., 2001; Rena et al., 2001). The 14-3-3 proteins mask the nuclear localisation signal (NLS) of FOXO3 to prevent nuclear translocation and serve as chaperone molecules to escort FOXO3 from the nucleus to the cytoplasm, where it is degraded via an ubiquitin-mediated process (Brunet et al., 1999; Brunet et al., 2001).

Low levels of the growth factors and insulin or oxidative stress, causes FOXO3 to become dephosphorylated and activated. The activated FOXO3 translocates to the nucleus where it acts to regulate the transcription of target genes (Murphy et al., 2003).

In the AMPK pathway, AMPK phosphorylation of FOXO3 is required to counteract the effects of nutrient deprivation and to induce stress resistance; a cellular function that is coupled with organismal longevity (Canto et al., 2009). The overall effect of AMPK activation is to switch off energy-consuming pathways and switch on energy-producing pathways. Greer et al. (2007) have shown that AMPK directly phosphorylates FOXO3 at six regulatory sites *in vitro* (Thr179, Ser399, Ser413, Ser555, Ser588, Ser626) (Figure 4.3 (c)) and at least two sites in cells (Ser413 and Ser588). In contrast with AKT phosphorylation, AMPK phosphorylation does not cause nuclear exclusion of FOXO3, but instead its activation (Greer et al., 2007). The exact mechanisms involved remain unclear, but it has been hypothesised that with five of the six phosphorylation sites being within the transactivation domain, this might make this domain more acidic, thereby increasing its transcriptional activation potential.

A genome-wide microarray analysis has shown that mutation of AMPK phosphorylation sites in FOXO3 specifically impairs the expression of a subset of target genes, including oxidative stress resistance and energy metabolism genes (Greer et al., 2007). This supports the contention that AMPK phosphorylation of FOXO3 appears to enhance the ability of transcription activity to up-regulate the expression of specific target genes. Therefore, the regulation of FOXO3 by AMPK may play a critical role in fine-tuning gene expression that controls energy balance and stress resistance in cells throughout their life.

- **The structure of the FOXO3 gene (*FOXO3*)**

The FOX family is characterised by having a conserved DNA-binding domain termed the “Forkhead” box (FOX) (Carlsson & Mahlapuu, 2002). Since the discovery of the first member of the family, the *Drosophila* Forkhead gene (*daf-16*); around 100 homologoues have been identified in species ranging from yeast to human (Carlsson & Mahlapuu, 2002; Gajiwala & Burley, 2000). FOX genes have recently been divided into 19 sub-families

(*FOXA* to *FOXS*) according to the amino acid sequences of their DNA-binding domains (Obsil & Obsilova, 2000).

In mammals, the FOXO sub-family contain four members: FOXO1 (FKHR), FOXO3 (FKHRL1), FOXO4 (AFX) and FOXO6. FOXO3 is the most abundant and most studied of the mammalian FOXO proteins. It is expressed in almost all cells and highly expressed in the heart, spleen, lung, kidney, ovary, adipose tissue and brain (Zhu et al., 2004).

The FOXO3 gene (*FOXO3*) typically consists of four exons and these produce a protein of a 673 amino acids (M. J. Anderson et al., 1998). From the N-terminus to the C-terminus, FOXO3 contains a proline-rich domain, a forkhead (FH) domain, a nuclear localisation signal (NLS), a nuclear export signal (NES), an LxxLL motif and a transactivation domain (Figure 4.3 (c)). The proline-rich motif binds to the CH_3 region of co-activators such as the CREB binding protein-p300 and stabilizes the interaction between FOXO3 and the co-activators (van der Heide & Smidt, 2005) .

The FH domain is composed of three major α-helices (H1-3), two large unstructured wings (W1, W2), and three β-strands (S1-3) (Figure 4.3 (a)) (F. Wang et al., 2008). The FH domain is specifically responsible for binding to the promoter region of target genes. The central region of FOXO3 includes the NLS and NES domains and accounts for the cellular localisation of FOXO3. The LxxLL motif is reported to bind to sirtuin (SIRT) and has an important role in regulating its transcriptional activity (Nakae et al., 2006). SIRT is a multifaceted enzyme that regulates a variety of cellular functions, from controlling gene expression and genome maintenance, to longevity and metabolism (Jiang, 2008; Pallás et al., 2008; D. Taylor et al., 2008). The C-terminus of FOXO3 contains the transactivation domain, which plays several important roles in DNA binding (Tsai et al., 2007).

The domain-like structure of FOXO3 suggests its activity is tightly regulated at different levels, including its intracellular translocation and transcriptional activity.

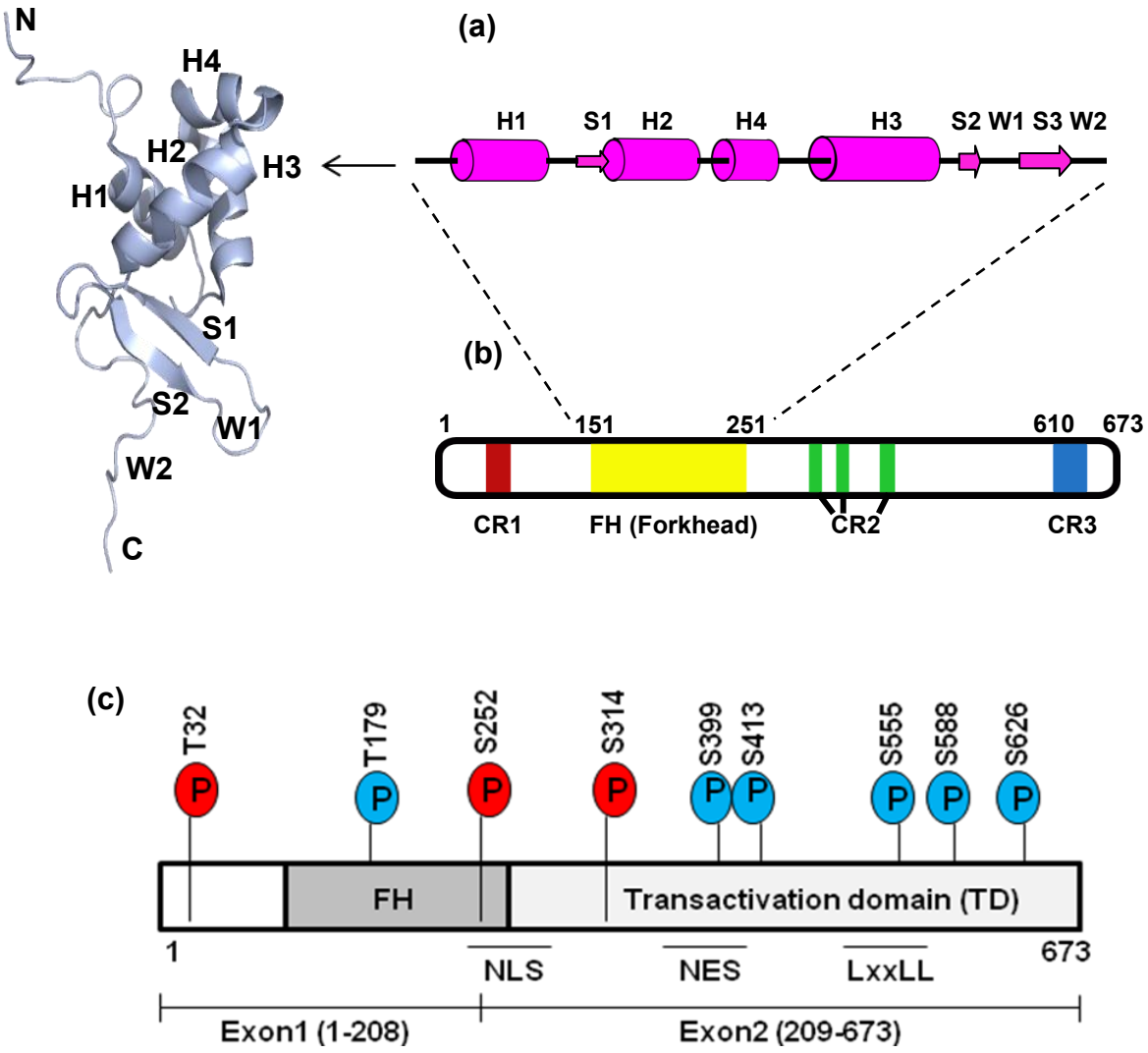


Figure 4.3. NMR structure and domain structure of FOXO3. (a) The secondary structure of the Forkhead (FH) domain derived from the NMR structure of the FH domain of FOXO3 (cylinders, α-helices; arrows, β-strands; lines, 1oops) is shown. (b) The domain-like structure of FOXO3 is shown with the well-folded FH domain and three other conserved regions, CR1-CR3, indicated. (c) The functional domain structure of FOXO3. The three amino acid residues that can be phosphoylated by PI3K-AKT (T32, S253 and S315) are indicated in red. The six AMPK amino acid residues are coloured blue; FH=Forkhead domain; X=any amino acid; NLS=nuclear localisaiton signal; NES=nuclear export signal (based on F.Wang et al., 2008)

- **Conservation of FOXO3 structure**

To date, the evolutionary conservation of FOXO genes (*FOXO1*, *FOXO3*, *FOXO4* and *FOXO6*) has been revealed across a diversity of species (M. Wang et al., 2009). Figure 4.4 is a phylogenetic tree based on the full-length coding sequences of FOXO genes from *Xenopus*, zebrafish, rodents, cows and humans (Figure 4.4). They all contain a forkhead domain (FH),

NLS and NES. They also share conserved post-translational modification sites. However, the physiological roles of the various FOXO proteins seem functionally diverse (Arden, 2008).

In *C. elegans* and *D. melanogaster*, the only FOXO transcription factor is encoded by DAF-16. A role for DAF-16 in promoting longevity has been established (Kenyon, 2005). The daf-16 gene is most similar to mammalian FOXO3 with the amino-acid sequences of DAF-16 and human FOXO3 being 27% identical over the full protein length and 82% identical within the forkhead DNA-binding domain (Jünger et al., 2003). Furthermore, Table 4.1 shows the amino acid sequence of FOXO3 is about 95% identical within mammals. This suggests that FOXO3 is highly conserved within mammalian species including sheep.

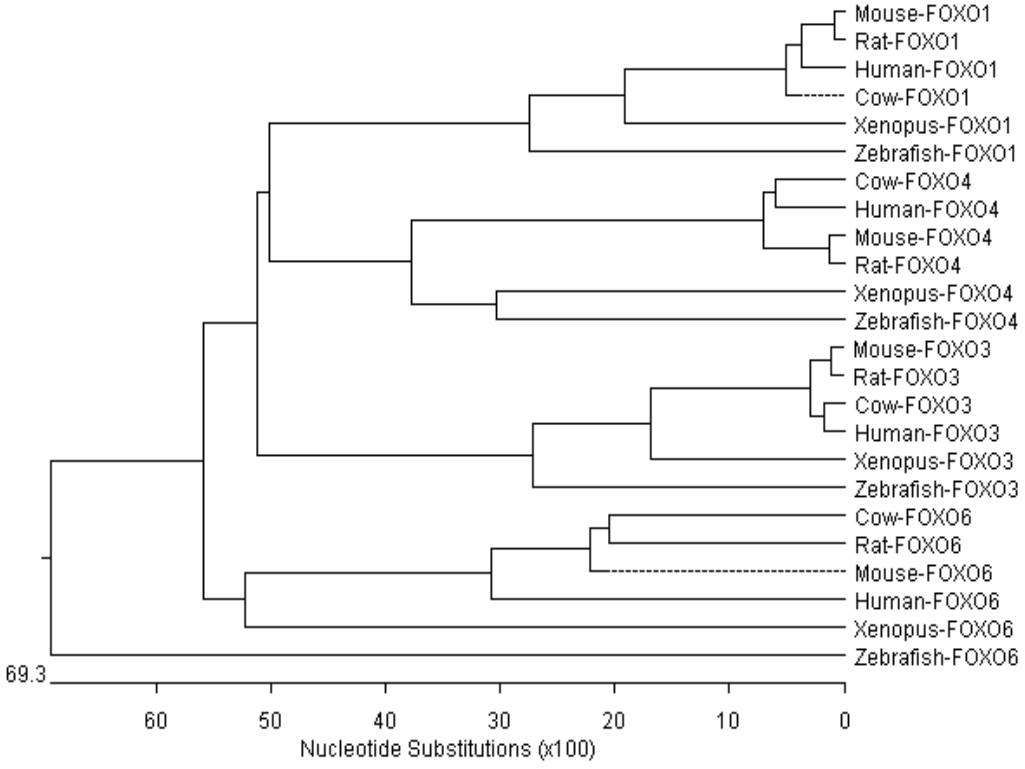


Figure 4.4. Phylogenetic tree of *FOXOs* from various species. The phylogenetic tree was constructed by the neighbour-joining method, using the multiple alignment algorithms in the MegAlign package (DNAStar Inc., Madison, WI, USA). The full-length nucleotide sequences of mRNA *FOXO3* were extracted from GenBank and Ensembl genome browsers (FOXO1: NP_002006.2 (*Homo sapiens*) for human; XM_002691748.1 (*Bos taurus*) for cow; CH466530.1 (*Mus musculus*) for mouse; NM_001191846.1 (*Rattus norvegicus*) for rat; NP_001086417.1 for *Xenopus laevis*; NP_001070725.2 for *Zebra fish*, FOXO3: NP_963853.1 (*Homo sapiens*) for human; gi|296484163 (*Bos taurus*) for cow; NP_062714.1 (*Mus musculus*) for mouse; NP_001099865.1(*Rattus norvegicus*) for rat; AAI70411.1 for *Xenopus laevis*; NP_571160.1 for *Zebra fish*, FOXO4: NP_005929.2 (*Homo sapiens*) for human; GJ063417.1 (*Bos taurus*) for cow; AL671926.11 (*Mus musculus*) for mouse; NP_001100413.1 (*Rattus norvegicus*) for rat; FJ811896.1 for *Xenopus laevis*; ENSDART00000078187 for *Zebra fish*, FOXO6: NP_002006.2 (*Homo sapiens*) for human; ENSBTAT00000033274 (*Bos taurus*) for cow; AAH86628.1 (*Mus musculus*) for mouse;

ENSRNOT00000050941 (*Rattus norvegicus*) for rat; NP_001152754.1 for *Xenopus laevis*; ENSDARP00000103866 for *Zebrafish*)

Table 4.1. Amino acid sequence identity for FOXO3 from various species.

(%) Identity	*Human*	*Cow*	*Mouse*	*Rat*	*Xenopus*	*Zebrafish*
Human	100					
Cow	96.4	100				
Mouse	94.8	94.2	100			
Rat	94.6	94.2	97.8	100		
Xenopus	71.2	70.9	70.6	70.4	100	
Zebrafish	55.8	57.5	55.8	56.7	56.4	100

- ***FOXO3* variation and variation in longevity and fertility**

Although studies in *C.elegans* and *D. melanogaster* point to there being a key role for FOXO3 in controlling life-span in these organisms (Partridge & Bruning, 2008), the role of the protein in mammalian aging has not yet been established. In humans, variant forms of *FOXO3* have been linked to longevity in seven populations located within different countries (Kuningas et al., 2006). For example, Willcox et al. (2008) first identified that *FOXO3* SNPs (rs2764264, rs13217795 and rs2802292) were strongly associated with longevity in long-lived (e.g., aged 95 and above) Americans of Japanese origin.

Flachsbart et al. (2009) confirmed this association using a large German population. They also genotyped 16 other polymorphisms of *FOXO3* and identified four SNPs (rs6911407, rs9400239, rs3800231 and rs479744) that were associated with longevity in all the German samples. *FOXO3* variation has also been linked to longevity in centenarians from southern Italian (Anselmi et al., 2009), Caucasians (Pawlikowska et al., 2009) and the Han Chinese (Y. Li et al., 2009).

Studies of animal models suggest FOXO3 has an evolutionarily conserved role in determining longevity (Fetterman et al., 2008). This would suggest that variation in *FOXO3* may influence longevity in sheep. In mice, variation in *FOXO3* has been implicated in the

effects of insulin on glucose metabolism, as well as on age-dependent female infertility (Castrillon et al., 2003; Hosaka et al., 2004). *FOXO3* mutant mice exhibit defects in glucose uptake (Castrillon et al., 2003), which may lead to age-related diseases such as diabetes and hypertension. Interestingly, *FOXO3*-null mice suffer from cell death in several cell types, especially ovarian follicles. These mice models have been shown to have accelerated premature ovarian failure (POF), suggesting that FOXO3 regulates follicular development. Several mutations within human *FOXO3* have been noted in women with POF (Gallardo et al., 2008; B. Wang et al., 2010; Watkins et al., 2006). These findings raised the possibility that mutations or naturally occurring variation in *FOXO3* might contribute to POF in other mammals and once again including sheep.

There are few studies on *FOXO3* variation in any livestock species, or whether this variation is associated with longevity and fertility. In this chapter, as no sequence information is available for sheep, sequences of the ovine *FOXO3* were firstly identified. For the genetic association study, the entire exon 2 of this gene was used to investigate genetic variation in sheep longevity. The second exon of *FOXO3* encodes part of the FH, CR2 and CR3 domains, including the DNA-binding sites and is a region where DNA sequences are highly conserved across species. If variation exists in this region of ovine *FOXO3*, then it might be useful for subsequent genetic studies into longevity and fecundity in sheep.

4.1 Materials and Methods

4.1.1 Sheep investigated to define variation in *FOXO3*

The whole coding sequence of ovine *FOXO3* and genetic variation in exon 2 of this gene were initially investigated in 182 New Zealand cross-bred sheep. Blood samples were collected from these animals onto FTA cards (Whatman BioScience, Middlesex, UK).

4.1.2 Sheep studied for longevity and fecundity analysis

Sheep blood samples along with longevity and fecundity data as described previously, were used (See chapter 2.2) to assess the effect of variation in *FOXO3* on sheep longevity and fecundity.

4.1.3 DNA extraction from blood on FTA cards

(See chapter 3.1.3)

4.1.4 PCR primer design and PCR-SSCP analysis

Six sets of PCR primers (Table 4.2) were designed based on the bovine *FOXO3* sequences (GenBank Accession No. XM_615634) to amplify the entire coding region of ovine *FOXO3*. These were synthesised by proligo (Boulder, CO. USA).

Table 4.2. Primers and annealing temperatures for ovine *FOXO3* amplification.

Primer pairs	Primer sequence (5'-3')	Primer location (nucleotide no.)[a]	Size (bp)[b]	(˚C)[c]
Exon 1				
Amplicon 1-up	GCGAAGATGGCAGAGGCGC	1-311	311	63
Amplicon 1-dn	CCTCCAGGAGCCAGCAGC			
Exon 1				
Amplicon 2-up	GCTCTGGGTTGCTCCTGG	263-622	360	63
Amplicon 2-dn	GCACCTTCCAGCAGGCAG			
Exon 2				
Amplicon 1-up	AACTCTATCCGGCACAACC	619-983	365	62
Amplicon 1-dn	GCCAGGATGGGGGACAG			
Exon 2				
Amplicon 2-up	AACGCCAGCACAGTCAGC	943-1362	420	64
Amplicon 2-dn	CTTGTTCTCTTGGATGGTCT			
Exon 2				
Amplicon 3-up	GACCGTCGTCTCTGAACTC	1304-1703	400	63
Amplicon 3-dn	TTGGCTGACCCGAGGCTG			
Exon 2				
Amplicon 4-up	CATGGGCTTGAGCGACTC	1665-2040	376	63
Amplicon 4-dn	TCCCCTTTCCTCAGTGAGC			
FOXO3-up	AGAGCTTATGGTGGATCATC	686-1165	480	62
FOXO3-dn (Genotyping PCR set)	GGTCGTCCATGAGGTTGTC			

[a] Nucleotide position is relative to the bovine *FOXO3* reference sequence (GenBank Accession No. XM_615634). [b] Expected amplicon size. [c] An annealing temperature for PCR.

Amplification was performed in a 20 μL reaction containing the washed genomic DNA on one 1.2-mm punch of FTA paper, 0.25 μM of each primer, 150 μM dNTPs (Eppendorf, Hamburg, Germany), 1.5 mM of $MgCl_2$, 0.5 U of Taq DNA polymerase (Qiagen, Hilden, Germany) and 1 × reaction buffer supplied. Amplification was carried out in an iCycler (Bio-Rad Laboratories, Hercules, CA, USA) and consisted of denaturation at 94 ºC for 2 min, followed by 35 cycles of 94 ºC for 30 s, the optimized annealing temperature for any given primer pair (Table 4.2) for 30 s, and 72 ºC for 30 s, with a final extension step at 72 ºC for 5 min. Amplicons were visualised by electrophoresis in 1% agarose (Quantum Scientific, Queensland, Australia) gels, using 1× TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM Na_2EDTA) containing 200 ng of ethidium bromide/mL.

A 0.7 μL aliquot of each amplicons was mixed with 7 μL of loading dye (98% formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene-cyanol), and after denaturation at 95 ºC for 5 min, samples were rapidly cooled on wet ice and then loaded on to 16 cm × 18 cm, 12% acrylamide:bisacrylamide (37.5:1: Bio-Rad) gels. Electrophoresis was performed using Protean II xi cells (Bio-Rad), at 200 V for 18 h at 25 ºC in 0.5 × TBE buffer. Gels were silver-stained according to the method of Byun et al. (2009).

4.1.5 Cloning of regions covering the *FOXO3* coding sequence

Sheep DNA samples that produced different SSCP patterns were selected for amplification using the proof-reading enzyme *Pwo* SuperYield DNA polymerase (Roche Applied Science, Mannheim, Germany) and using the conditions described previously. Amplicons were ligated into the pCR4Blunt-TOPO vector (Invitrogen, Carlsbad, CA), and a 2 μL aliquot of the ligation mixture was used to transform competent *Escherichia coli* cells (One Shot INVαF′, Invitrogen), following the manufacturer's instructions. Ten insert positive white colonies for each transformation were picked and incubated overnight in Terrific broth (Invitrogen) at 37 ºC, in a shaking rotary incubator (225 rpm). Plasmid DNA was recovered from bacterial cells by boiling for 10 min in 0.8% (vol/vol) Triton X-100, followed by centrifugation at 12,000 × g. A 1 μL aliquot of the supernatant was used as a template for PCR amplification using *Taq* DNA polymerase (Qiagen). Amplicons from these clones and the corresponding genomic DNA were run adjacent to each other on SSCP gels for comparison of the SSCP patterns, and only those clones for which the SSCP patterns matched those of the corresponding original genomic DNA sample were selected for subsequent DNA sequencing.

4.1.6 DNA sequencing and sequence analysis

Plasmid DNA was extracted from overnight cultures using a QIAprep Spin Miniprep Kit (Qiagen) and was sequenced in both directions using the M13-forward and reverse primers. Identical sequences obtained from at least three separate clones from sheep were sequenced at Lincoln University, New Zealand.

Sequence alignments, translations, phylogenetic tree, analyses and other comparisons were carried out using DNASTAR (Madison, WI, USA) and DNAMAN (Version 5.2.10, Lynnon BioSoft, Vaudreuil, Canada). The BLAST algorithm was used to search the NCBI GenBank (http://www.ncbi.nlm.nih.gov/) and Ensembl (http://www.ensembl.org) databases for homologous sequences to those that were amplified.

4.1.7 *FOXO3* haplotype determination

As the exon 2 region of *FOXO3* encodes part of FH and the transactivation domain, (including the DNA-binding sites and other conserved regions), only this exon was screened to identify if genetic variation exists and whether that genetic variation is associated with variation in longevity.

To characterise the entire exon 2 region of ovine *FOXO3*, four fragments were amplified using four separate sets of PCR primers (Table 4.2). Haplotypes were determined by screening to find sheep homozygous for any one of the given amplicons, and then by analysing those same sheep at the other amplicons to ascertain what sequence variation extended through that region.

4.1.8 Genotyping of ovine *FOXO3* for longevity and fecundity trait analysis

For the association study, a different genotyping primer set (table 4.2, FOXO3-up and FOXO3-dn) was designed subsequently based on the seven haplotypes of ovine *FOXO3* that were identified initially. Sheep DNA samples were amplified using the new primer set and amplicons were subject to PCR-SSCP analysis in 12% acrylamide:bisacrylamide (37.5:1: Bio-Rad) gels at 200 V for 18 h at 25 ºC in 0.5 × TBE buffer.

The genetic association study was carried out on 1732 ewes from seven breeds including NZ Romney (n = 324), Corriedale (n = 316), Merino (n = 314), Polwarth (n = 107), Kelso (n = 171), Texel (n = 40) and Coopworth (n = 460) selected from 37 different flocks. Overall, there were nine hundred and sixty-three older ewes and seven hundred and sixty-nine

young ewes (2 years old). Lambing information was available for six hundred and thirty-one of the older ewes.

4.1.9 Statistical analyses

Data was analysed using SPSS version 17 (SPSS Science Inc., Chicago, IL, USA), with a two-tailed significance level of $\alpha = 0.05$.

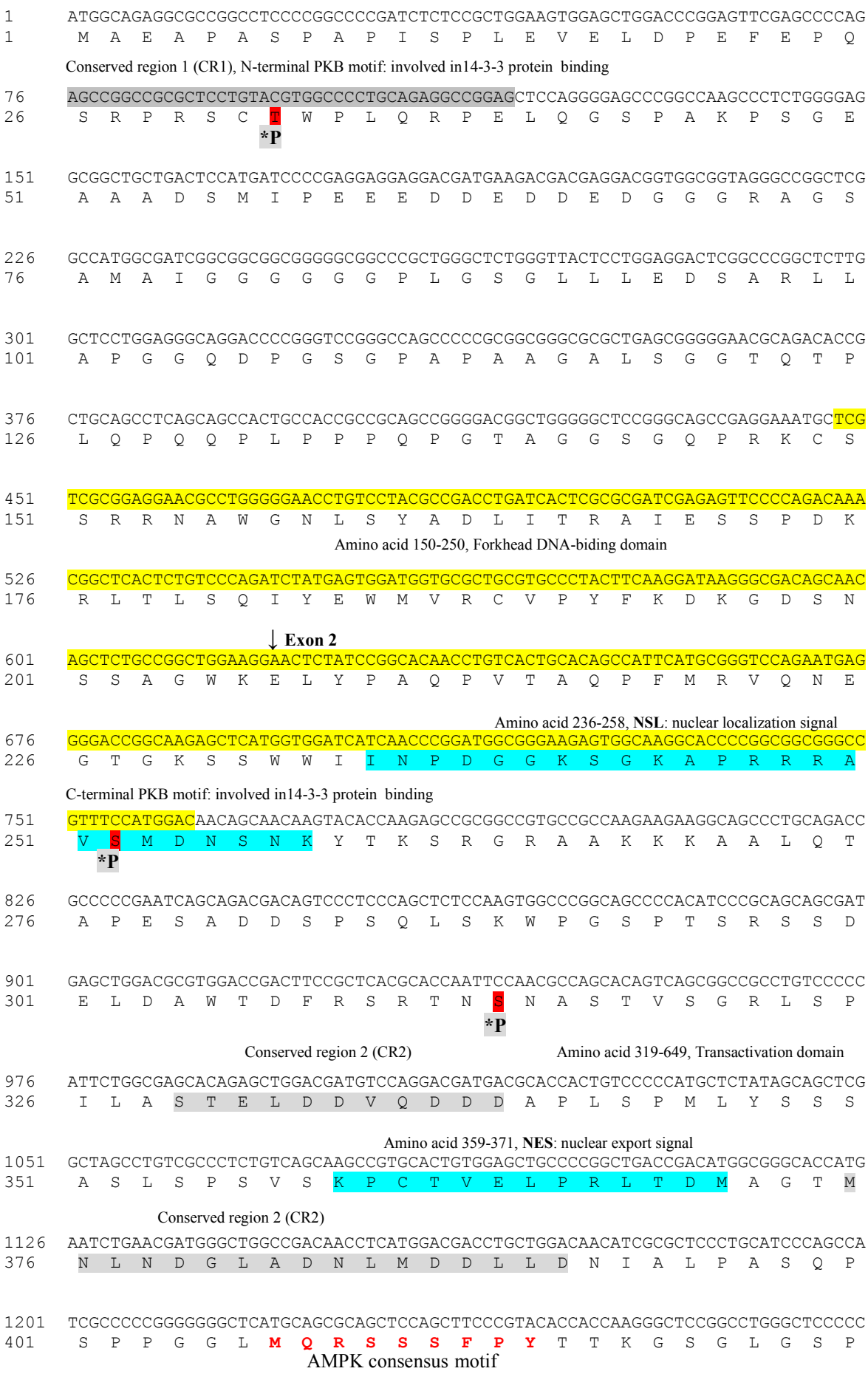
Unadjusted analyses exploring the relationship between *FOXO3* haplotypes (*A* to *G*) and longevity were undertaken using a one-way analysis of variance (ANOVA). Any haplotypes showing significant associations with longevity from these analyses were then further analysed using a general linear mixed model (GLMM) to assess whether the effects of each of the *FOXO3* haplotypes on longevity, persisted when breed and farm effects were entered into the models.

4.2 Results

4.2.1 Identification of the coding sequence of ovine *FOXO3*

Using six sets of primers designed based on exon 1 and 2 of bovine *FOXO3* (Table 4.2), six overlapping DNA fragments were amplified and sequenced. Two of these fragments produced a 618 bp sequence covering all of exon 1, while four produced a 1400 bp sequence for exon 2. These sequences were assembled to produce a 2019 bp fragment using the bovine sequence as a template for assembly and the fragment deposited into the NCBI GenBank with the accession number JQ 894783. The construct covered the whole coding sequence of ovine *FOXO3*. Figure 4.5 shows the entire coding sequence of ovine *FOXO3* (JQ 894783) and the putative amino acid sequence that it would produce.

The putative amino acid sequence derived for ovine *FOXO3* was aligned with amino acid sequences from cattle (GenBank Accession No. NP_001193012), human (GenBank Accession No. NP_001446) and *Xenopus laevis* (GenBank Accession No. NP_001086418) (Figure 4.6). The alignment revealed that this gene is conserved across species.


1 ATGGCAGAGGCGCCGGCCTCCCCGGCCCCGATCTCTCCGCTGGAAGTGGAGCTGGACCCGGAGTTCGAGCCCCAG
1 M A E A P A S P A P I S P L E V E L D P E F E P Q
Conserved region 1 (CR1), N-terminal PKB motif: involved in14-3-3 protein binding
76 AGCCGGCCGCGCTCCTGTACGTGGCCCCTGCAGAGGCCGGAGCTCCAGGGGAGCCCGGCCAAGCCCTCTGGGGAG
26 S R P R S C T W P L Q R P E L Q G S P A K P S G E
*P
151 GCGGCTGCTGACTCCATGATCCCCGAGGAGGAGGACGATGAAGACGACGAGGACGGTGGCGGTAGGGCCGGCTCG
51 A A A D S M I P E E E E D D E D D E D G G G R A G S
226 GCCATGGCGATCGGCGGCGGCGGGGGCGGCCCGCTGGGCTCTGGGTTACTCCTGGAGGACTCGGCCCGGCTCTTG
76 A M A I G G G G G G P L G S G L L L E D S A R L L
301 GCTCCTGGAGGGCAGGACCCCGGGTCCGGGCCAGCCCCCGCGGCGGGCGCGCTGAGCGGGGGAACGCAGACACCG
101 A P G G Q D P G S G P A P A A G A L S G G T Q T P
376 CTGCAGCCTCAGCAGCCACTGCCACCGCCGCAGCCGGGGACGGCTGGGGGCTCCGGGCAGCCGAGGAAATGCTCG
126 L Q P Q Q P L P P P Q P G T A G G S G Q P R K C S
451 TCGCGGAGGAACGCCTGGGGGAACCTGTCCTACGCCGACCTGATCACTCGCGCGATCGAGAGTTCCCCAGACAAA
151 S R R N A W G N L S Y A D L I T R A I E S S P D K
Amino acid 150-250, Forkhead DNA-biding domain
526 CGGCTCACTCTGTCCCAGATCTATGAGTGGATGGTGCGCTGCGTGCCCTACTTCAAGGATAAGGGCGACAGCAAC
176 R L T L S Q I Y E W M V R C V P Y F K D K G D S N
↓ Exon 2
601 AGCTCTGCCGGCTGGAAGGAACTCTATCCGGCACAACCTGTCACTGCACAGCCATTCATGCGGGTCCAGAATGAG
201 S S A G W K E L Y P A Q P V T A Q P F M R V Q N E
Amino acid 236-258, **NSL**: nuclear localization signal
676 GGGACCGGCAAGAGCTCATGGTGGATCATCAACCCGGATGGCGGGAAGAGTGGCAAGGCACCCCGGCGGCGGGCC
226 G T G K S S W W I I N P D G G K S G K A P R R R A
C-terminal PKB motif: involved in14-3-3 protein binding
751 GTTTCCATGGACAACAGCAACAAGTACACCAAGAGCCGCGGCCGTGCCGCCAAGAAGAAGGCAGCCCTGCAGACC
251 V S M D N S N K Y T K S R G R A A K K K A A L Q T
*P
826 GCCCCCGAATCAGCAGACGACAGTCCCTCCCAGCTCTCCAAGTGGCCCGGCAGCCCCACATCCCGCAGCAGCGAT
276 A P E S A D D S P S Q L S K W P G S P T S R S S D
901 GAGCTGGACGCGTGGACCGACTTCCGCTCACGCACCAATTCCAACGCCAGCACAGTCAGCGGCCGCCTGTCCCCC
301 E L D A W T D F R S R T N S N A S T V S G R L S P
*P
Conserved region 2 (CR2)
Amino acid 319-649, Transactivation domain
976 ATTCTGGCGAGCACAGAGCTGGACGATGTCCAGGACGATGACGCACCACTGTCCCCCATGCTCTATAGCAGCTCG
326 I L A S T E L D D V Q D D D A P L S P M L Y S S S
Amino acid 359-371, **NES**: nuclear export signal
1051 GCTAGCCTGTCGCCCTCTGTCAGCAAGCCGTGCACTGTGGAGCTGCCCCGGCTGACCGACATGGCGGGCACCATG
351 A S L S P S V S K P C T V E L P R L T D M A G T M
Conserved region 2 (CR2)
1126 AATCTGAACGATGGGCTGGCCGACAACCTCATGGACGACCTGCTGGACAACATCGCGCTCCCTGCATCCCAGCCA
376 N L N D G L A D N L M D D L L D N I A L P A S Q P
1201 TCGCCCCCGGGGGGGCTCATGCAGCGCAGCTCCAGCTTCCCGTACACCACCAAGGGCTCCGGCCTGGGCTCCCCC
401 S P P G G L M Q R S S S F P Y T T K G S G L G S P
AMPK consensus motif

```
1276  ACCAGCTCCTTCAGCAGCGCGGTATTTGGTCCCTCGTCTCTGAACTCCCTGCGCCAGTCTCCCATGCAGACCATC
426    T  S  S  F  S  S  A  V  F  G  P  S  S  L  N  S  L  R  Q  S  P  M  Q  T  I

                                                                  Conserved region 2 (CR2)
1351  CAAGAGAACAAGCCAGCCACCTTCTCTTCCATGTCCCATTATGGCAACCAGACACTCCAGGACCTGCTCACGTCG
451    Q  E  N  K  P  A  T  F  S  S  M  S  H  Y  G  N  Q  T  L  Q  D  L  L  T  S
                                                                  LXXLL

1426  GACTCACTCAGCCACAGCGATGTCATGATGACCCAGTCGGACCCCTTGATGTCTCAGGCCAGCACCGCTGTGTCC
476    D  S  L  S  H  S  D  V  M  M  T  Q  S  D  P  L  M  S  Q  A  S  T  A  V  S

1501  GCCCAGAACTCCCGCCGGAACGTGATGCTTCGCAGTGACCCAATGATGTCCTTTGCCGCCCAGCCTAACCAGGGG
501    A  Q  N  S  R  R  N  V  M  L  R  S  D  P  M  M  S  F  A  A  Q  P  N  Q  G

1576  AGTTTGGTCAATCAGAACTTGCTCCACCACCAGCACCAAACCCAGGGCGCTCTCGGTGGCAGCCGTGCCTTGTCG
526    S  L  V  N  Q  N  L  L  H  H  Q  H  Q  T  Q  G  A  L  G  G  S  R  A  L  S

1651  AATTCCGTCAGCAACATGGGCTTGAGCGACTCCAGCAGCCTCGGGTCAGCCAAACACCAGCAACAGTCTCCCGTC
551    N  S  V  S  N  M  G  L  S  D  S  S  S  L  G  S  A  K  H  Q  Q  Q  S  P  V

1726  AGCCAGTCTATGCAAACCCTCTCGGACTCTCTCTCAGGCTCCTCCTTGTACTCAACCAGTGCGAACCTTCCAGTC
576    S  Q  S  M  Q  T  L  S  D  S  L  S  G  S  S  L  Y  S  T  S  A  N  L  P  V

                                  Amino acid 609-649, the conserved region 3 (CR3)
1801  ATGGGCCACGAGAAGTTCCCCAGCGACTTGGACCTGGACATGTTCAATGGGAGCTTGGAATGTGACATGGAGTCC
601    M  G  H  E  K  F  P  S  D  L  D  L  D  M  F  N  G  S  L  E  C  D  M  E  S

1876  ATTATCCGTAGTGAACTCATGGATGCTGATGGGTTGGATTTTAACTTTGATTCCCTCATCTCCACACAGAATGTT
626    I  I  R  S  E  L  M  D  A  D  G  L  D  F  N  F  D  S  L  I  S  T  Q  N  V

1951  GTTGGTTTGAACGTGGGGAGCTTCACTGGTGCTAAGCAGGCCTCATCTCAGAGCTGGGTGCCAGGCTGA
651    V  G  L  N  V  G  S  F  T  G  A  K  Q  A  S  S  Q  S  W  V  P  G  •
```

Figure 4.5. Nucleotide and predicted amino acid sequence of ovine *FOXO3*. The nucleotide and predicted amino acid sequences (2019 bp and 672 amino acids, respectively) of ovine *FOXO3* are shown with numbering indicated at the beginning of each line. The position of the stop codon is indicated by an "•". Symbol (P*) denotes possible sites for PKB phosphorylation based on the human protein (Tsai et al., 2007; Greer et al., 2007). LXXLL motif- L; leucine, X; any amino acid

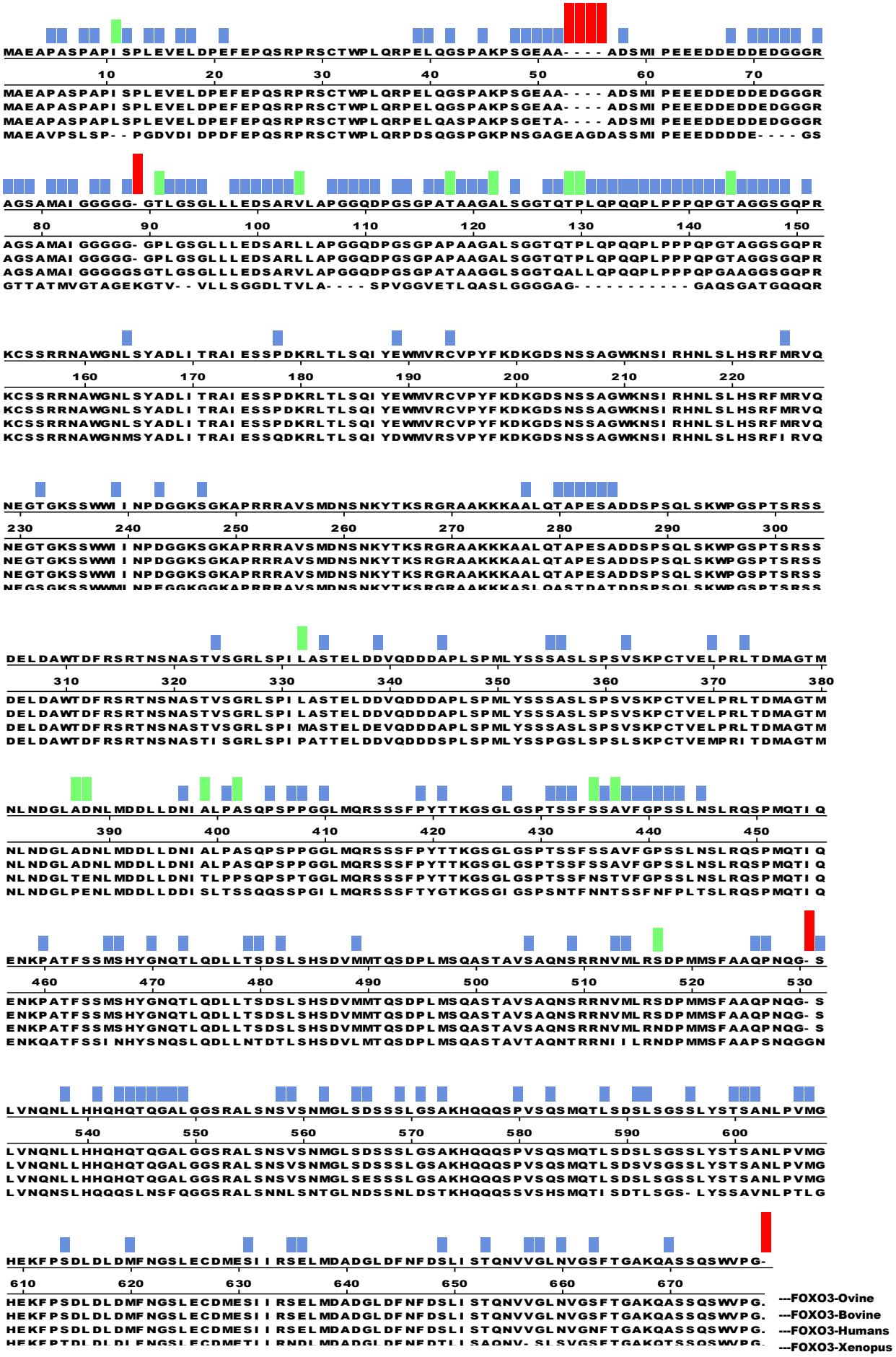


Figure 4.6. Amino acid alignment of sheep, cattle, human and toad FOXO3.

4.2.2 Identification of seven haplotypes for ovine *FOXO3*

Variation in exon 2 of ovine *FOXO3* was identified from 182 NZ cross-bred sheep using four separate sets of PCR primers (Table 4.2).

Upon SSCP analysis, six and three distinct banding patterns were detected in amplicon 1 and 2, respectively (Figure 4.7). No SSCP variation was detected in amplicon 3 and 4 (Figure 4.7). Either one, or two distinct banding patterns were observed for each sheep, which defines homozygous or heterozygous genotypes, respectively. Novel variants from amplicon 1 and 2 were sequenced, while there was only one sequence obtained from amplicon 3 and 4. All the sequences identified shared high homology to the published bovine *FOXO3* sequence (GenBank Accession No. XM_615634), suggesting that these sequences represented variant forms of ovine *FOXO3*.

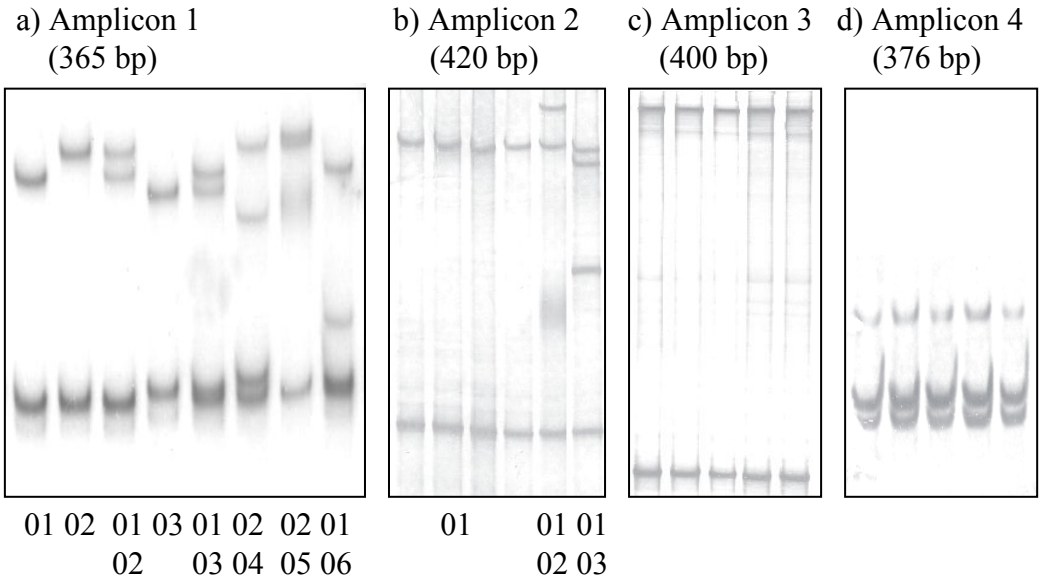


Figure 4.7. PCR-single-strand conformational polymorphism of four fragments within exon 2 of ovine *FOXO3*. Six and three unique SSCP patterns corresponding to six and three variant sequences (01-06 and 01-03) are shown for amplicons 1 and 2, respectively, while no SSCP variation was detected in amplicons 3 and 4

Seven extended haplotypes were assembled from the four amplicons (Table 4.3). The seven haplotypes detected were named *A* to *G* and deposited into the NCBI GenBank with the accession numbers GQ995521 to GQ995527. Sequence analysis revealed ten single nucleotide substitutions in exon 2 (Table 4.4). One of the substitutions, an A to G substitution at nucleotide position 601, leads to a putative amino acid substitution of M (methionine) with

V (valine) at position 201 (position 407 of the bovine sequence NCBI GenBank reference sequence XM_002690117). It is notable that this methionine residue is highly conserved in a diversity of species (Figure 4.8).

Table 4.3. The seven haplotypes derived from amplicon 1 and 2 of ovine *FOXO3*.

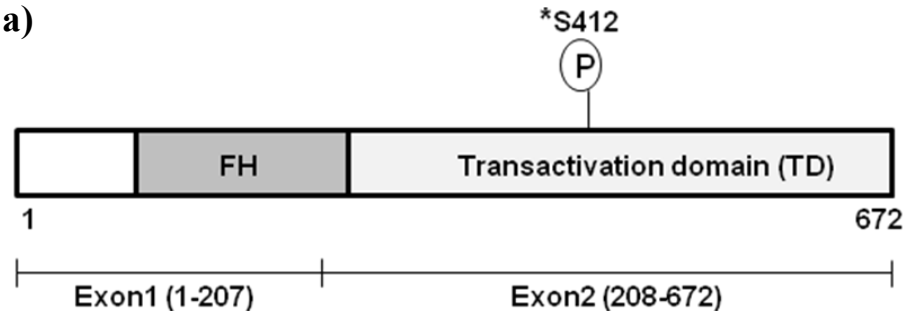
Haplotype	Amplicon 1 — Amplicon 2 (365 bp) (420 bp)	Accession Numbers
A	01 —— 01	GQ995521
B	02 —— 01	GQ995522
C	02 —— 03	GQ995523
D	03 —— 02	GQ995524
E	04 —— 01	GQ995525
F	05 —— 01	GQ995526
G	06 —— 01	GQ995527

Table 4.4. Sequence variation in exon 2 of ovine *FOXO3*.

SNP	Position[1]	Haplotype *A*	Haplotype *B*	Haplotype *C*	Haplotype *D*	Haplotype *E*	Haplotype *F*	Haplotype *G*	Amino acid substitution
G/A	93	G	G	G	G	A	G	G	–
C/T	99	C	C	C	C	C	C	T	–
T/C	135	T	T	T	C	T	T	T	–
C/T	207	C	T	T	C	C	T	C	–
A/G	216	A	A	A	G	A	A	A	–
C/T	258	C	C	C	C	C	T	C	–
C/T	327	C	C	C	C	C	T	C	–
C/A	489	C	C	A	C	C	C	C	–
T/C	519	T	T	T	C	T	T	T	–
A/G	601	A	A	A	**G**	A	A	A	M/V
	Frequency[2] (%)	49.0	30.0	0.2	14	0.1	0.3	0.1	

[1] Nucleotide position is given relative to the first nucleotide of exon 2 of a bovine *FOXO3* reference sequence (GenBank Accession No. XM_615634). [2] In 182 New Zealand cross-bred sheep.

a)



b)

AMPK consensus motif: Hyd-(X, Basic)XXS/TXXX-Hyd

	407 412	
	* *	
Sheep (haplotype D)	QPSPPGGLVQRSSSFPY	(GQ995524)
Cattle	QPSPPGGLMQRSSSFPY	(ref seq XM 002690117)
African Bush Elephant	QTSPPGALMQRSSSFPY	(ref seq XM_003404305)
Pig	QPSPPGGLMQRSSSFPY	(ref seq NM 001135959)
Horse	QPSPPGGLMQRSSSFPY	(ref seq XM 001503987)
Giant panda	QPSPTGGLMQRSSSFPY	(ref seq XM_002917347)
Dog	QPSPTGGLMQRSSSFPY	(ref seq XM 863298)
Rhesus monkey	QPSPTGGLMQRSSSFPY	(ref seq XM_001093593)
Common marmoset	QPSPTGGLMQRSSSFPY	(ref seq XM 002746951)
Sumatran orang-utan	QPSPTGGLMQRSSSFPY	(ref seq XM 002817219)
Chimpanzee	QPSPTGGLMQRSSSFPY	(ref seq XM_003311514)
Gray short-tail opossum	QPSPSAGLMQRSSSFPY	(ref seq XM 001368456)
Chicken	QQSPTGGMMQRSSSFPY	(ref seq XM_001234495)
Turkey	QQSPTGGIMQRSSSFPY	(ref seq XR 118137)
Xenopus sp.	QQSSPGVLMQRSSSFTY	(ref seq NM 001092949)
Platypus	QPSPSAGLLQRSATFPY	(ref seq XM_001511115)

Figure 4.8. The location of ovine FOXO3 AMPK site and the AMPK consensus motif. a) The AMPK phosphorylation site of ovine *FOXO3* at Ser412 b) Alignment of putative amino acid sequences from NCBI GenBank reference sequences showing that the hydrophobic methioine (residue 407 of the bovine sequence) is highly conserved across species. The consensus recognition motif for AMPK is Hyd-(X, Basic)XXS/TXXX-Hyd (where Hyd=M,L,I,F or V, Basic=R>K>H and S/T=phosporylation site)

4.2.3 Development of a simpler *FOXO3* genotyping method

Based on the DNA sequences obtained for the seven extended haplotypes, a new set of PCR primers (FOXO3-up and FOXO3-dn, Table 4.2) were designed. These gave unique and identifiable PCR-SSCP patterns that spanned the variable region of exon 2 and that could be matched to the original seven haplotypes (*A* to *G*) (Figure 4.9). This genotyping method allowed establishment of a simple and reliable method for typing large numbers of sheep.

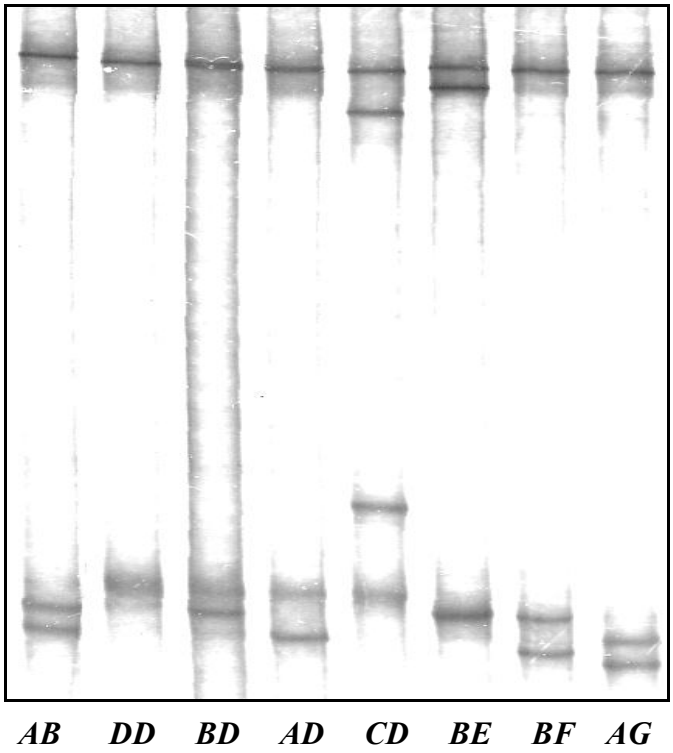


Figure 4.9. The PCR-SSCP typing system developed for ovine *FOXO3*. Seven unique SSCP patterns corresponding to seven different haplotypes of the ovine *FOXO3* are shown

4.2.4 Genetic association with sheep longevity and fecundity

A total of 1732 ewes were typed using the improved genotyping method. The haplotype frequencies are shown in Table 4.5. Haplotypes *C*, *E*, *F* and *G* were rare (< 2%) and a univariate analysis revealed no significant association between them and longevity (Table 4.5).

In an analysis, without adjustment for breed and flock, three haplotypes (*A*, *B* and *D*) of *FOXO3* showed significant associations with sheep age ($P < 0.01$, data not shown). However, when sheep carrying these three haplotypes were adjusted for either breed or flock in a

GLMM, only the *D* haplotype remained significantly associated with longevity (Table 4.5). When corrected for breed, the presence of *D* decreased age by about five months with a mean age of 5.0 ± 0.2 years, whereas ewes that did not possess the haplotype had a mean age of 5.5 ± 0.1 years ($P = 0.006$). When corrected for the flock, the presence and absence of the *FOXO3 D* haplotype had a mean age of 4.7 ± 0.1 years and 5.1 ± 0.1 years ($P = 0.034$), respectively (Table 4.5).

No *FOXO3* haplotypes were found to have any effect on fecundity in either flock or breed corrected models (Table 4.6).

Table 4.5. Association of ovine *FOXO3* exon 2 variation and longevity.

Haplotype	Frequency (%)[1]	Haplotype status	n	Age means (Breed adjusted)	Age means (Flock adjusted)
A	45.3	Present	1268	5.4 ± 0.1	5.0 ± 0.1
		Absent	464	5.5 ± 0.2	5.1 ± 0.2
				($P = 0.490$)	($P = 0.562$)
B	30.4	Present	693	5.4 ± 0.1	4.9 ± 0.1
		Absent	1039	5.4 ± 0.1	5.1 ± 0.1
				($P = 0.966$)	($P = 0.240$)
C	1.0	Present	24	5.8 ± 0.6	5.1 ± 0.6
		Absent	1708	5.4 ± 0.1	5.0 ± 0.1
				($P = 0.536$)	($P = 0.829$)
D	21.7	Present	486	5.0 ± 0.1	4.7 ± 0.1
		Absent	1246	5.5 ± 0.1	5.1 ± 0.1
				($P = 0.006$)	**($P = 0.034$)**
E	1.2	Present	69	5.1 ± 0.4	4.8 ± 0.4
		Absent	1663	5.4 ± 0.1	5.0 ± 0.1
				($P = 0.430$)	($P = 0.667$)
F	0.3	Present	14	6.5 ± 0.8	6.0 ± 0.8
		Absent	1718	5.4 ± 0.1	5.0 ± 0.1
				($P = 0.169$)	($P = 0.187$)
G	0.1	Present	21	6.4 ± 0.1	5.9 ± 0.7
		Absent	1711	5.4 ± 0.7	5.0 ± 0.1
				($P = 0.138$)	($P = 0.180$)

[1] Rounded frequencies in 1732 New Zealand sheep.

Table 4.6. Association of ovine *FOXO3* exon 2 variation and fecundity.

Haplotype	Haplotype status	n	Fecundity means (Breed adjusted)	Fecundity means (Flock adjusted)
A	Present	176	1.8 ± 0.02	1.8 ± 0.02
	Absent	455	1.8 ± 0.03	1.8 ± 0.03
			(*P* = 0.328)	(*P* = 0.190)
B	Present	215	1.8 ± 0.03	1.8 ± 0.03
	Absent	416	1.8 ± 0.02	1.8 ± 0.02
			(*P* = 0.243)	(*P* = 0.675)
C	Present	8	1.8 ± 0.13	1.9 ± 0.12
	Absent	623	1.8 ± 0.02	1.8 ± 0.01
			(*P* = 0.664)	(*P* = 0.398)
D	Present	186	1.8 ± 0.03	1.8 ± 0.03
	Absent	445	1.8 ± 0.02	1.8 ± 0.02
			(P = 0.371)	(P = 0.621)
E	Present	23	1.9 ± 0.08	1.8 ± 0.08
	Absent	608	1.8 ± 0.02	1.8 ± 0.01
			(*P* = 0.616)	(*P* = 0.905)
F	Present	8	2.0 ± 0.13	2.0 ± 0.12
	Absent	623	1.8 ± 0.02	1.8 ± 0.01
			(*P* = 0.297)	(*P* = 0.152)
G	Present	11	2.0 ± 0.12	1.9 ± 0.11
	Absent	620	1.8 ± 0.02	1.8 ± 0.01
			(*P* = 0.328)	(*P* = 0.342)

4.3 Discussion

The evolutionarily conserved FOXO3 transcription factor is a major target of insulin-like signalling, and is therefore important in the regulation of development, metabolism and longevity (Murphy, 2006).

This is the first report of an association between variation in ovine *FOXO3* and variation in life-span in sheep. In this study, the coding sequence of ovine *FOXO3* was firstly identified and compared with other species. Moreover, the sequence comparison revealed a high level of similarity within mammals. This supports the contention that there is an evolutionarily conserved role for FOXO3, including a likely role in controlling longevity in sheep.

The entire coding region of the FOXO3 gene is contained within exon 1 and 2. Exon 2 encompasses the DNA-binding domain of the transcription factor, including part of the FH domain and conserved regions (CR) 2 and 3. Recent NMR and biochemical studies have demonstrated that the FH and CR domains play a central role in regulating DNA interaction (Tsai et al., 2007), hence variation in this region of the gene may influence FOXO3 activity. Accordingly I investigated exon 2 of ovine *FOXO3* to ascertain if it was genetically variable, and if so, whether this variation was associated with variation in longevity.

In this study, ten SNPs defining seven haplotypes (named *A* to *G*) were identified in the 1422-bp exon 2 fragment of ovine *FOXO3* that was assembled (Table 4.3). In haplotype *D*, an adenine to guanine substitution at nucleotide position 601 of exon 2 leads to a putative amino acid substitution of methionine to valine at residue 201 (residue 407 of the bovine sequence NCBI GenBank reference sequence XM_002690117). It is notable that the substitution in haplotype *D* is located at the hydrophobic site of an AMPK consensus binding motif. In mammals, FOXO3 contains an AMPK consensus phosphorylation motif, which is Hyd-(X,Basic)XXS/TXXX-Hyd, where Hyd represents a hydrophobic residue, X represents any amino acid residue, basic represents a basic residue and S/T represents a serine or threonine (see the Figure 4.8). In the AMPK motif, a phosphorylation site (a serine or threonine residue) is flanked by hydrophobic and basic residues, and the sequence around the phosphorylation site is highly conserved across species. Generally, the consensus hydrophobic residue is methionine, but it can also include leucine, isoleucine, phenylalanine and valine. In haplotype *D*, the hydrophobic methionine is substituted by valine, which is also hydrophobic, but the methionine is highly conserved within a diversity of species (Figure 4.8), the exception being the egg-laying

monotreme platypus. This suggests the methionine residue is under some conformational and/or functional constraint.

In humans, FOXO3 is phosphorylated by AMPK at Thr179, Ser399, Ser413, Ser555, Ser588 and Ser626 (these would correspond to sheep Thr178, Ser398, Ser412, Ser554, Ser587 and Ser625). Among the six AMPK phosporylation sites, activation of FOXO3 at Ser412 by AMPK appears to enhance the expression of genes implicated in cellular stress responses and longevity (Greer et al., 2007). In this study, the *D* haplotype of the ovine *FOXO3* defined by variation in the consensus AMPK motif surrounding Ser412, was significantly associated with a decreased mean age for the sheep. This supports the contention that the *D* haplotype may be important in determining FOXO3 transcriptional activity.

It has been reported that a point mutation in murine *Foxo3a* exon 2 results in an amino acid substitution (I 181 N), and that this is associated with a premature loss of fertility due to abnormal early primordial follicle activation and subsequent abnormal folliculogenesis (Youngson et al., 2011). Fertility loss has also been observed in a homozygous *Foxo3a* "knockout" mouse model and is caused by an early depletion of functional follicles (Castrillon et al., 2003). Thus the variation observed in this study in ovine exon 2 may affect FOXO3 functionality, and in turn, reproductive ability. This may increase the likelihood of sheep being culled as a consequence of not becoming pregnant at mating. This could explain why the sequence variation in haplotype *D* is associated with a decreased life-span in these stud sheep.

The rest of the genetic variation detected in this study are synonymous SNPs and therefore potentially functionally neutral (Sharp et al., 1995). However, recently there has been mounting evidence against this line of thought. For example, some reports suggest that synonymous substitutions affect mRNA secondary structure and stability, and hence they may have functional effects (Parmley et al., 2006; Qu et al., 2006). The apparently silent polymorphism detected here could therefore also be functionally important and affect FOXO3 activity. They may also be in linkage with variation elsewhere in the gene that affects its expression.

In farmed-sheep systems, life-span is controlled by death rates and culling decisions. Farmers' cull their breeding stock for a variety of reasons. These include their reproductive performance, genetic performance, propensity to ill-health, incisor wear, age and combinations of these factors. Variation in the value of products that will be derived from the

sheep is also a key reason for culling and given market variation in product prices, culling decisions are also sensitive to market and season.

McGregor (McGregor, 2011) argues that sheep farmers use the wear and loss of permanent incisors as a major reason for culling sheep and it appears that both farmers and scientists regard the impact of permanent incisor wear and loss on the production and premature wastage of ewes as an inevitable outcome of advancing age in sheep. Accordingly, in commercial sheep production systems, casting sheep for age is often driven by knowledge of when permanent incisor condition is likely to deteriorate. In one New Zealand study, 65% of farmers culled ewes because of incisor faults and there was additional culling of ewes with faulty incisors because of their poor body condition (Orr et al., 1986). However, despite the widespread practices of culling adult sheep at the entry of replacement ewes to the breeding flock; and the casting of sheep for age; there are surprisingly few objective reports of the reasons for culling ewes, or of the effectiveness of culling. Hence, in the absence of clear objective evidence, New Zealand farmers have generally adopted the "safe" path of casting ewes at a particular age.

This was one of the reasons why stud ewes were chosen in this study. As a consequence of their enhanced value, breeding sheep are frequently retained longer in flocks than commercial sheep and therefore are less likely to have simply been culled based on their age. This is a requirement for studying ewe longevity, as age-based culling in a commercial system would likely mask any under-lying genetic effects on life-span. What-is-more, while incisor loss may be pronounced in these stud sheep, their maintenance in a stud is likely to be more affected by their ongoing reproductive success and genetic merit.

Given the variation in longevity between flocks and within a given breed, farm-related factors are a major determinant of longevity. Accordingly "farm" needs to be incorporated into any statistical analysis of life-span. However most farms in this study only have one breed of sheep and hence, farm and breed are confounded. Accordingly, breed and flock were corrected for in separate models. Given that the reasons for culling sheep out of flocks are diverse and potentially inter-related, it is nevertheless conceivable that factors like variation in fertility or resistance to disease may also explain the association we have found.

Four of the haplotypes are not commonly found suggesting that they offer little productive advantage in the sheep studied. The reason for their low frequency can only be speculated upon, but would not preclude them also affecting longevity, fertility and/or other

production traits. In conclusion, genetic variability in *FOXO3* may potentially affect sheep longevity either directly, or indirectly by impacting on factors that lead to reduced productivity and increased likelihood of culling. This requires further study.

Chapter 5
An investigation of variation in ovine *CAST* and its association with longevity and fecundity

Calpastatin (CAST) is the only known endogenous inhibitor of calpain and is ubiquitously expressed. Calpains refer to a family of intracellular, calcium-dependent cysteine proteases, which comprises several tissue-specific isoforms (n-calpains) as well as two ubiquitous isozymes (μ- and m-calpains or calpain I and II, respectively) (Goll et al., 2003). These proteins have been identified in nearly every animal cell type studied, but the amount of each protein varies from tissue to tissue (Goll et al., 2003). CAST is generally found at a higher concentration than the calpains.

The calpains and CAST constitute the calpain system, which is involved in a variety of cellular processes, including cytoskeletal remodelling, cellular signalling, apoptosis and cell survival (Rivett, 1990; Sorimachi et al., 2011; Sorimachi et al., 1997).

Calpains are totally dependent on Ca^{2+} for their catalytic activity and function in various cellular activities (Goll et al., 2003). μ- and m-calpain, which have different Ca^{2+} requirements (micromolar and milimolar concentration, respectively) are well studied in mammals. Both μ- and m-calpains are heterodimers, consisting of distinct 80-kDa large subunits and a 30-kDa regulatory subunit (Figure 5.1).

In general, the calpains exist in the cytosol as inactive enzymes and translocate to membranes in response to increased cellular calcium levels. At the membrane, calpain is activated in the presence of calcium and phospholipids (Khorchid & Ikura, 2002). Activated calpain cleaves a variety of protein substrates at hinge regions including between neighbouring functional and regulatory domains. This leads to activation, inactivation, or destruction of the substrate. Over 100 calpain substrates have been identified including cytoskeletal proteins (desmin, α-actinin, vimentin, spectrin, integrin, cadherin), membrane proteins (growth factor receptors, adhesion molecules, ion transporters), enzymes (kinases, phospholipases, phosphatases), myofibrillar proteins (troponin, tropomyosin, myosin light chain kinase), as well as cytokines and various transcription factors and other proteins (Goll et al., 1983). It also acts upon proteins involved in apoptotic signalling (Squìer et al., 1994).

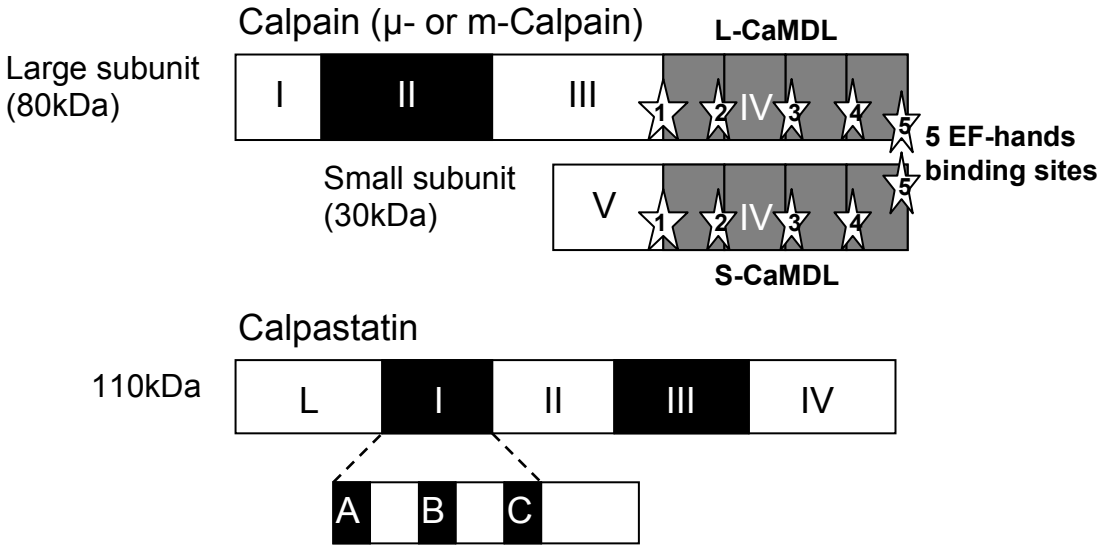


Figure 5.1. Schematic domain representation of calpain and calpastain. Calpain domains I to IV make up the large 80 kDa subunit and the small 30 kDa subunit comprises domains V and VI. Protease activity is localised to domain II, domains IV and VI are the calcium binding segments that are implicated in the activation and regulation of the enzyme. 5 potential calcium binding EF-hands (penta-EF hands, the major Ca2+ binding domains) are represented as stars. Calpastatin sub-domain B is responsible for calpain inhibition, sub-domain A and C bind to the calmodulin-like domain IV and VI of the calpain large subunit (L-CaMLD) (adapted from Goll et al., 2003)

Calpain therefore plays an important regulatory role in cellular processes, including the remodelling of cytoskeletal proteins, the control of signal-transduction pathways, the degradation of enzymes controlling progression through the cell-cycle, the regulation of gene expression and control of apoptotic pathways. Inappropriate calpain activation could therefore result in various pathological states, leading to irreversible alterations and loss of functionality for the calpain substrate. Furthermore, an over-activated calpain system has been shown to participate in a number of pathological conditions including hypoxia, ischemia, spinal cord injury, Alzheimer disease, muscular dystrophy and cataract formation (Bertipaglia & Carafoli, 2007; Zatz & Starling, 2005).

As the activation of calpain is an irreversible reaction, calpain activation must be strictly controlled, particularly in neurons, which are vulnerable and exhibit limited regeneration. CAST regulates calpains by inhibiting both the proteolytic activity of the enzymes and their binding to membranes (Goll et al., 2003).

In mammalian cells, there are known to be eight different isoforms of CAST, resulting from both alternative splicing of the primary transcript and proteolytic processing. Most of the CAST isoforms are expressed ubiquitously and only some of them are restricted to particular organs and cell types (Melloni et al., 1998; Melloni et al., 1992). It is suggested that different CAST isoforms inhibit different calpain isoforms within a single cell (Averna et al., 1999). Table 5.1 summarises the splicing sites found in the CAST gene and describes how the sites are important for generating different CAST isoforms in specific physiological roles.

CAST has an internal repeat of four homologous domains which allows it to simultaneously inhibit multiple calpain molecules. Each inhibitory domain has three subdomains, called A, B, and C (Figure 5.1). The three subdomains of CAST mediate calpain inhibition. Sub-domains A and C bind to the calmodulin-like domain IV and VI of the calpain large subunit (L-CaMLD), respectively, while B binds to domain II of calpain which contains the protease activity (Figure 5.1). Thus, interaction between the inhibitory domain of CAST and calpain is essential for calpain activity, and this suggests the regulation of calpain activity is tightly controlled by CAST.

Although the calpain system has been implicated in regulating a number of cellular processes, including longevity (Nixon et al., 1994), there has been no specific study of the potential influence of CAST on longevity in sheep. In this chapter, the role of CAST in longevity was investigated as well as an investigation of its roles in muscle growth and fecundity.

- **A role for CAST in longevity?**

Changes in the rate or efficiency of proteolysis have featured in many hypotheses on the mechanism of aging. Calpain activity specifically is of interest in regulation of cellular protein metabolism and homeostasis. Lynch et al. (1986) first showed that calpain activity may be related to the rate of aging. They found that members of the Chiroptera order (Bats), that have extended life-spans, also have decreased calpain activity in the brain (five to sevenfold lower) when compared with its activity in other similar-sized mammals, like mice.

Calpain activity increases in cells and tissues during normal aging. For example, in mammals, an overactive calpain system has been associated with a variety of age-related disorders, including Alzheimer and Parkinson diseases, cerebral ischemia, ischemic myocardial infarction, spinal cord injury, muscular dystrophy, rheumatoid arthritis and cataract formation (Higuchi et al., 2005; Karlsson et al., 1995; K. K. Wang & Yuen, 1994).

The process of cataract formation is a particularly well-established degenerative phenomenon of aging. This process seems to result from the specific proteolytic cleavage of α-crystallin, leading to its denaturation (David et al., 1992). In vertebrate eyes, α-crystallin is a major structural protein of the lens and plays an important role in maintaining its transparency by preventing the aggregation or inactivation of several proteins (Groenen et al., 1994; Horwitz, 2003; Reddy et al., 2000).

It has been suggested that aging reflects deterioration of cell cytoskeletal elements (Halbhuber et al., 1987; Kay et al., 1989; Kelley & Perdue, 1980). Calpains are also known to regulate many interactions between the membrane and the intra-cellular environment (Nixon, 1989), and this could play a role in cell differentiation, exfoliation and aging. CAST is the only natural inhibitor of both μ–and m–calpains, thus during aging, calpain activity in protein degradation is regulated by CAST. This suggests a role for CAST in the aging process (Goll et al., 2003). In mammals, several lines of evidence also suggest that CAST is implicated in age-related pathologies (Averna et al., 2007; Vaisid et al., 2007). Rao et al.(2008) provided evidence that CAST is a regulator of constitutive calpain activity in the process of neural aging. Working with Alzheimer disease brains, transgenic mice and other cell models, they demonstrated that CAST depletion can accelerate calpain-mediated activation of specific protein kinases and cytoskeleton disruption. This can ultimately lead to cell death. Furthermore, they have shown that over-expression of CAST in transgenic mice, suppressed calpain activity and created protective effects on the onset of aging in neurons (Rao et al., 2008). This suggests that the life-span of animals might be regulated by the expression of CAST.

Although CAST is now thought to play a role in regulating longevity, its function in livestock has primarily been studied in relation to meat quality, and specifically meat tenderness (Kemp et al., 2010). To date, there has been only one study suggesting that genetic variation in *CAST* may affect longevity in dairy cattle (Garcia et al., 2006). In the research described here *CAST* is investigated as gene that might affect longevity in sheep.

- **A role for CAST in fecundity?**

Several studies of the expression of CAST in reproductive tissues and organs such as the human pituitary gland (Eto et al., 1995), the human placenta (Thompson et al., 2002), the human oocyte (Ben-Aharon et al., 2005), the bovine corpus luteum (Orwig et al., 1994) and the mouse testis (Li & Goldberg, 2000), have suggested that CAST may play an important role in mammalian reproduction.

During fertilisation in mammals, the sperm cell triggers a unique signal-transduction mechanism within the egg, leading to its activation (Yanagimachi & Bhattacharyya, 1988). This event requires membrane fusion between the sperm cell and the oocyte. Sperm calpain is associated with the cell membrane fusion process that takes place during penetration of the oocyte (Rojas et al., 1999). Thus, given CAST's role elsewhere in regulating calpain, it might participate in regulating the breakdown of cytoskeletal proteins during fertilisation.

In addition, research on fertilisation in rats and humans has revealed that the calpain system may play a role in regulating mammalian oocyte activation. For example, Ben Aharon et al. (2005) and Haim et al. (2006) have suggested that CAST mediates rat oocyte activation. Ben-Aharon et al. (2005) also reported the expression of the calpain-calpastatin system in human oocytes.

A recent study has investigated genetic variation in bovine *CAST* and it was suggested that CAST may influence fertility (Garcia et al., 2006). Taken together this would suggest that *CAST* is a worthy candidate for studying in relation to fecundity in sheep.

- **A role for CAST in muscle growth and regulation of birth weight?**

In cattle (Chung et al., 1999; Ciobanu et al., 2004), and pigs (Koćwin-Podsiadła et al., 2003; Z. Li et al., 2009), *CAST* is reported to affect various muscle traits, including weight. The role of calpain in muscle protein-turnover is determined by the balance between muscle protein synthesis and muscle protein degradation and is important for muscle growth (Goll et al., 1992). Since muscle growth is the major determinant of the performance of meat-producing animals, many animal scientists have focussed their research on increasing the rate of muscle growth by increasing muscle protein synthesis, or decreasing muscle protein degradation.

The calpain system is essential for fetal growth and prenatal skeletal muscle development (myogenesis) (Du et al., 2004; Wu et al., 2006), and this may therefore affect birth weight traits. In the livestock industry, weight traits are important factors, because the process of early organization of muscles greatly influences an animal's growth as well as wool production in sheep specifically.

Expression of the calpain system has been negatively correlated with birth weight and fractional rate of growth (Z. Li et al., 2009; van den Hemel-Grooten et al., 1997). Z. Li et al. (2009) have shown that decreased levels of calpain expression during neonatal development in pigs are associated with higher protein accumulation. This suggests that protein

accumulation is related to a reduction in the proteolytic capacity of the muscle and largely due to increased CAST activity. Thus, CAST activity is involved in the control of muscle growth and birth weight traits.

- **The structure of CAST**

In mammals, CAST is translated to produce two main isoforms, a 110-kDa muscle tissue type and a 70-kDa erythrocyte type (Goll et al., 2003; Takano et al., 2000) Typical CAST molecules have non-inhibitory N-terminal domains (domain-L and domain-XL) and four repetitive inhibitory domains (domain I to domain IV) (Figure 5.2). In mice, the *CAST* isoforms (I to IV) consist of 29 coding exons, and the first exon of each type of *CAST* started lxa, lxb, 1u and 14t (Table 5.1) (Takano et al., 2000).

The CAST binding region is localised in the L-domain containing the amino acid sequences encoded by exons 2-8 (Melloni et al., 2006). Recently, the L domain of CAST has been shown to have a pivotal role in regulating Ca^{2+} channels (Minobe et al., 2006). Exons 4-7 of domain L contain sequences that are involved in the regulation of Ca^{2+}, specifically to induce calpain-CAST interaction (Melloni et al., 2006). This suggests that the exons encoding CAST domain L could be an important regulator of calpain activity.

Cong et al. (1998) have identified an additional CAST N-terminal peptide sequence (XL, 68 amino acids) in bovine heart and some of liver tissues. These are phosphorylated by protein kinase A (PKA) (Figure 5.2). They also found that the XL domain plays a regulatory role in altering phosphorylation patterns in the protein.

The individual inhibitory domains of CAST (domain I to domain IV) inhibit both μ–and m-calpains, but the ability of these domains to inhibit the calpains has been reported to vary in the following order: domain I> domain IV> domain III> domain II, from most to least effective (Kawasaki et al., 1989). Within each inhibitory domain there exists three highly conserved sequences designated A, B and C (Goll et al., 2003). Regions A and C bind calpain in a strictly Ca^{2+}-dependent manner, but have no inhibitory activity; whereases region B inhibits calpain on its own as it contains a well-conserved sequence, GxxE/DxTIPPxYR which is thought to be involved in exerting the inhibitory function (Figure 5.2).

Table 5.1. The genetic structure of mouse *CAST* isoforms. (Takano et al., 2000)

CAST isoform type	Exon number	Splicing acceptor site	Coding length (bp)	Splicing donor site	Intron length (kb)
Type I	1xa	---	75	gtagaggtag	4.3
	1y	atatttttag	63	gtaatttctg	3.8
	1x	tgtttctaag	72	gtcagtcaat	3.4
Domain	2	tcctttctag	60	gtaaagaaac	3.9
L	3	cttctattag	57	gtgatgtagt	0.6
	4	cccactttag	39	gtaagtaggt	1.5
	5	gaaattacag	54	gtgagtgata	0.5
	6	tgcacgtcag	114	gtgagcttga	1.3
	7	ctgctctcag	90	gtgagcatac	3.4
	8	ctccctctag	69	gtatggagcc	1.2
1	9	ctatttctag	99	gtatgtgttg	1.5
	10	tttcttccag	81	gtaagttatc	0.6
	11	tttggttcag	39	gtgagttgaa	0.8
	12	ttttctctag	87	gtattgattt	0.1
	13	tctttttag	90	gtacttaaat	1.6
2	14	tttttaacag	102	gtaatgactt	3.3
	15	ttttcatcag	84	gtaagcgctc	1.3
	16	ttattacag	48	gttggagaat	2.0
	17	cttcttacag	90	gtagaagtat	1.2
	18	cttttctcag	96	gtgagtgatg	1.8
3	19	gctcttgcag	102	gtaatggcag	0.5
	20	ttttatttag	84	gtaacaggct	3.2
	21	ctctgaatag	51	gtaagcaatc	0.2
	22	cttcttgcag	72	gtaagtatac	6.9
	23	ttaataaaag	99	gtaagcaaca	3.0
4	24	taccttttag	105	gtaaaagatc	1.0
	25	atgtttgtag	93	gtaaactaat	1.0
	26	tatttttaag	36	gtaaatacta	0.5
	27	acatgttaag	93	gtacctacac	2.0
	28	attgctttag	69	gtaaacatgg	3.5
	29	tccccctag	51	---	
Type II	1xb	---	30	gtaaatagaa	3.7
Type III	1u	---	-	gtgagtactg	1.1
Type IV	14t		165	gtgcacatta	2.1
			120	gtaatatagct	2.1

The *CAST* genomic DNA sequences are based on Acc. No. AB044310-AB044334

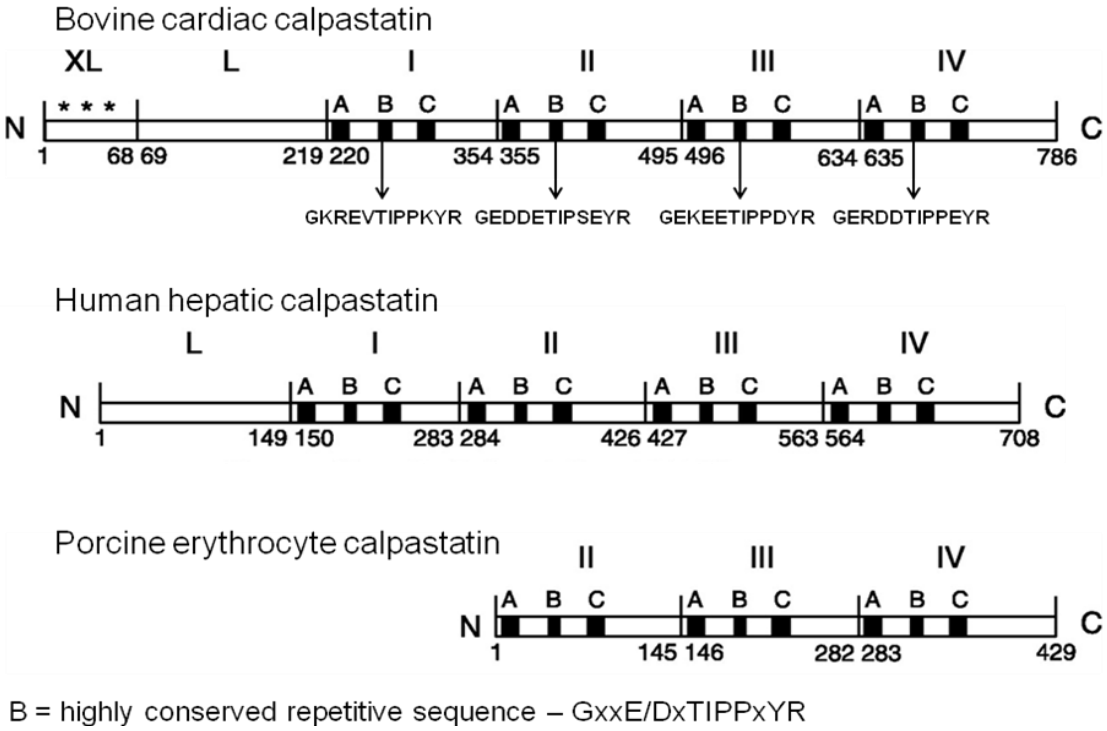


Figure 5.2. Schematic diagrams of three different calpastatin isoforms. Schematic diagram shows the domain structure of three different calpastatin isoforms. The calpastatin isoforms containing an L and/or XL domain are found in a number of tissues from a number of different species. A, B and C are three subdomains within each inhibitory domain and the B subdomain from a number of species contains the conserved sequence indicated. Three sites on domain XL that are phosphorylated by protein kinase A are marked,*** (adapted from Goll et al., 2003)

- **Variation in *CAST***

A number of variants forms of the CAST gene that affect muscle growth traits have been described in cattle (Barendse et al., 2007) and pigs (Ciobanu et al., 2004), but little is known about the effect of these variants and longevity and fecundity.

Variation in *CAST* in cattle has been the subject of several studies. These have detected variation in both the coding (Chung et al., 1999) and non-coding regions (Schenkel et al., 2006), as well as the 3' un-translated region (Casas et al., 2006). To date, in sheep, variation in the ovine *CAST* has been reported and in both exon 6 (H. Zhou et al., 2007a) and intron 12 (Palmer et al., 1998; Roberts et al., 1996). Five and three variant sequences respectively, have been identified in exon 6 and intron 12 of the ovine gene. Exon 6 encodes part of the CAST L domain, which is involved in the regulation of Ca^{2+}-induced proteolysis. This suggests that variation within exon 6 might affect the function of CAST and therefore potentially birth weight, muscle growth, longevity and fecundity traits. In this chapter, variation in this region

was assessed in sheep that were of varying age and for which fecundity records were available.

As haplotypes of multiple markers typically have a higher information content compared with single markers for assessing the genetic basis of traits, haplotypic diversity of the ovine *CAST* was also investigated. This involved studying the extended haplotyes defined by variation in both *CAST* exon 6 and intron 12.

5.1 Materials and methods

5.1.1 Sheep investigated to define haplotypic diversity

To investigate haplotypic diversity of ovine *CAST*, 1200 New Zealand cross-bred sheep (Romney, Coopworth and Perendale) were studied. These sheep were randomly selected from around New Zealand.

5.1.2 Sheep studied for birth weight and growth rate analysis

A total of 519 NZ Romney lambs from 17 sire lines were investigated. Birth weight, birth rank (single or multiple birth) and weaning weight were recorded. Growth rate was expressed as weight gain per day, which is the difference between weaning weight and birth weight, divided by age in days. Gender was also recorded (see appendix K for the data).

5.1.3 Sheep studied for longevity and fecundity analysis

Blood samples from sheep with longevity and fecundity data were used (See chapter 2.2 for sheep studied and the collection of longevity and fecundity data) to assess the effect of variation in *CAST* on longevity and fecundity.

5.1.4 DNA extraction from blood on FTA cards

(See chapter 3.1.3)

5.1.5 PCR primer design and synthesis

Exon 6 of ovine *CAST* was amplified using the primers described previously by H. Zhou et al. (2007). These were 5'-GTTATGAATTGCTTTCTACTC-3' and 5'-ATACGATTGAGAGACTTCAC-3' and their location is shown in Figure 5.3. In order to study haplotypic diversity within ovine *CAST*, intron 12 of ovine *CAST* was also investigated in conjunction with the genotyping of exon 6. As variation in intron 12 of this gene has been reported previously by Roberts et al. (1996), two primers specific for this region were

designed based on the sequences of the bovine (GenBank accession no. L14450) (Killefer & Koohmaraie, 1994), and ovine CAST genes (Roberts et al., 1996, AF016006-AF016008). These primers were 5'-CTCCCACTTTAAGACAACAAC-3' and 5'-AGCCTCTTCTGTAGATTTCTG-3' (See Figure 5.3). The BLAST algorithm was used to search the National Centre for Biotechnology Information (NCBI) GenBank (http://www.ncbi.nlm.nih.gov/) for homology and ensure the primers were specific to *CAST*. Primer secondary structures, primer dimerisation, primer melting temperatures and GC content were analysed using DNAMAN (Version 5.0, Lynnon BioSoft, Vaudreuil, Canada). The primers were synthesised by Integrated DNA Technologies (Coralville, IA, USA). Figure 5.3. shows the two regions of primer binding in ovine *CAST*.

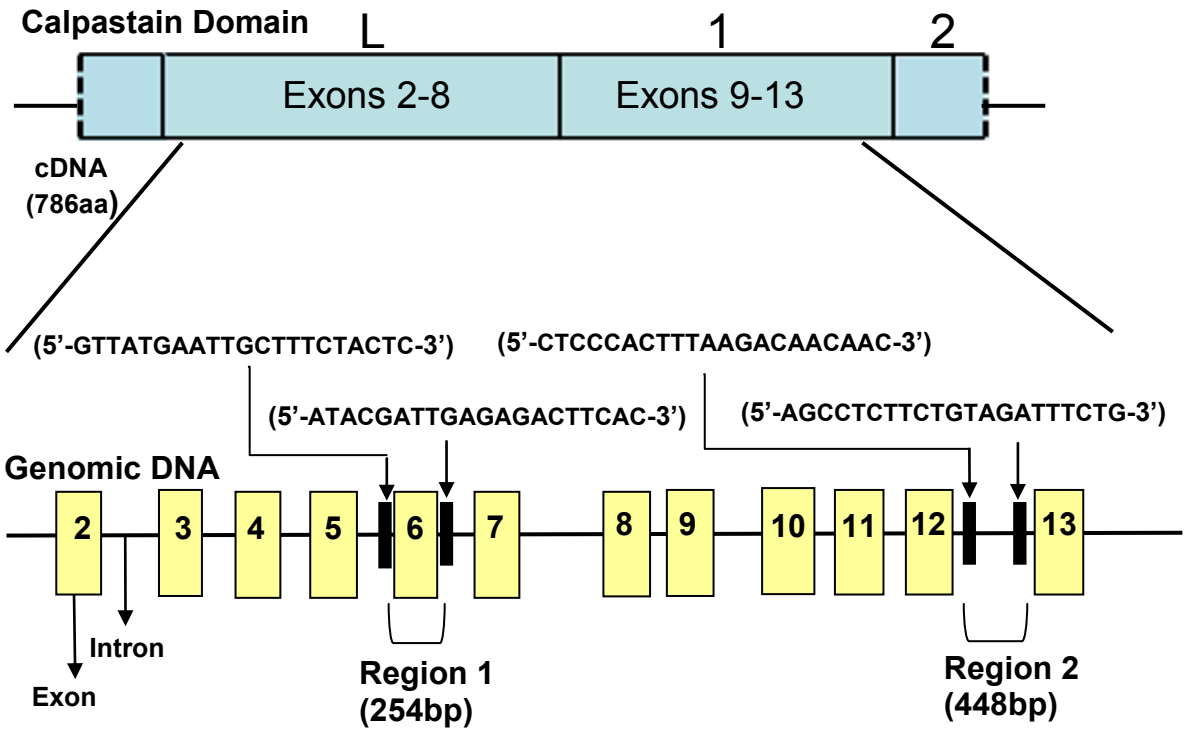


Figure 5.3. The location of PCR primers designed to amplify two regions of ovine *CAST*. The gene structure is based on bovine *CAST* sequences (GenBank accession no. AY 834770 and AY834771). The light blue region represents a schematic structure of bovine *CAST*. Domain L and 1 are shown. The exons that encode these domains are shown in light yellow. Primer binding sites for exon 6 and intron 12 are indicated. Amplicon 1 contains part of intron 5, intron 6 and the entire exon 6 of ovine *CAST*, while amplicon 2 contains part of intron 12

5.1.6 PCR-SSCP analysis

Amplification was performed in a 20-μL reaction containing the genomic DNA on one 1.2-mm punch of FTA paper, 0.25 μM of each primer, 150 μM of dNTPs (Eppendorf, Hamburg, Germany), 2.5 mM Mg^{2+}, 0.5 U of *Taq* DNA polymerase and 1 × the reaction buffer supplied. Amplification was carried out in an iCycler (Bio-Rad Laboratories, Hercules, CA) and consisted of denaturation at 94 ºC for 2 min, followed by 35 cycles of 94 ºC for 30 s, 55 ºC (amplicon 1-exon 6) or 60 ºC (amplicon 2-intron 12) for 30 s, and 72 ºC for 30 s, with a final extension step at 72 ºC for 5 min. Amplicons were visualised by electrophoresis in 1% agarose (Quantum Scientific, Queensland, Australia) gels, using 1 × TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM Na_2EDTA) containing 200 ng of ethidium bromide/mL.

A 0.7 μL aliquot of each amplicon was mixed with 7 μL of loading dye (98% formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene-cyanol) and after denaturation at 95 ºC for 5 min, samples were rapidly cooled on wet ice and then loaded on to 16 cm × 18 cm, 12% (amplicon 1-exon 6) or 8% (amplicon 2-intron 12) acrylamide:bisacrylamide (37.5:1: Bio-Rad) gels. Electrophoresis was performed using Protean II xi cells (Bio-Rad), at either 270 V at 12 ºC (amplicon 1-region 1) or 390 V at 4 ºC (amplicon 2-region 2), for 18 h in 0.5 × TBE buffer. Gels were silver-stained according to the method of Sanguinetti (1994).

5.1.7 Haplotype determination

The PCR-SSCP method does not determine specific haplotypes, but instead reveals what variant forms of the exon 6 and intron 12 regions a sheep carries. However, using the PCR-SSCP technique, haplotypes were indirectly determined. A population of 1200 samples from New Zealand cross-bred sheep was genotyped at exon 6 (amplicon 1), and sheep homozygous at exon 6 were subjected to genotyping at intron 12 (amplicon 2). Haplotypes through the two regions were then determined based on inference from the genotypes of the exon 6 homozygous individuals. Amplicons representative of the known *CAST* sequences were included in each polyacrylamide gel, and their banding patterns were used as standards for identification of individual samples.

5.1.8 Genotyping of ovine *CAST* exon 6 for birth weight, growth rate, longevity and fecundity analysis

For the genetic association study, sheep for which birth weight, growth rate, longevity and fecundity data were available were studied. This included 519 NZ Romney lambs for which birth and growth rate data were available and 1726 ewes from six breeds for which

longevity and fecundity data were available. These included NZ Romney (n = 340), Corriedale (n = 318), Merino (n = 326), Polwarth (n = 129), Kelso (n = 174) and Coopworth (n = 439) selected from 36 different flocks. Overall there were nine hundred and fifty-eight older ewes and seven hundred and sixty-eight young ewes (2 years old). Lambing information (fecundity data) was available for six hundred and fifty-eight of the older ewes.

5.1.9 Statistical analyses

Data was analysed using SPSS version 17 (SPSS Science Inc., Chicago, IL, USA), with a two-tailed significance level of $\alpha = 0.05$.

To assess whether any of the exon 6 *CAST* sequences (*A* to *E*) affected birth weight and growth rate, an ANOVA was performed using the significance level of $\alpha = 0.05$ to assess the effect of variant sequence presence (or absence), sire and birth rank (as well as the interactions of these factors) on birth weight and growth rate. To investigate the relationship between birth weight and growth rate to weaning, a Pearson Correlation was performed.

A general linear mixed model (GLMM) was used to assess the effect of the presence/absence of each of the exon 6 *CAST* sequences (*A* to E) on longevity and fecundity. These analyses were run as unadjusted models and additionally, in separate models adjusting for flock and breed effects, which were included as random factors.

5.2 Results

Haplotyping all the sheep available in this study would have been too costly financially and in time. Accordingly, exon 6 of ovine *CAST* was chosen for the genetic association study. Exon 6 is the most variable and spans the L domain, which is involved in the regulation of Ca^{2+} channels and calpain activity. Figure 5.4 shows five unique banding patterns corresponding to five unique sequences DNA of ovine *CAST*.

A simple and efficient PCR-SSCP method for genotyping part of intron 12 of ovine CAST gene was developed, and using this method in conjunction with the genotyping of exon 6, haplotypic diversity over an extended region of *CAST* could be analysed.

Using the primers designed for intron 12 (Figure 5.3), amplicons of the expected size (approximately 448 bp) were obtained. These amplicons were subjected to SSCP analysis, and four distinct banding patterns were obtained (Figure 5.5). Previously, Roberts et al (1996) had described three sequences at intron 12, but the amplicon that was used to reveal these sequences was too large for optimal use in PCR-SSCP. Therefore new primers within this

region were designed to amplify a smaller fragment which included all the known polymorphic sites and which resulted in the detection of not just the three previously reported sequences, but a new sequence as well (Table 5.2). The newly identified sequence has been deposited into the NCBI GenBank with accession No. EF669476.

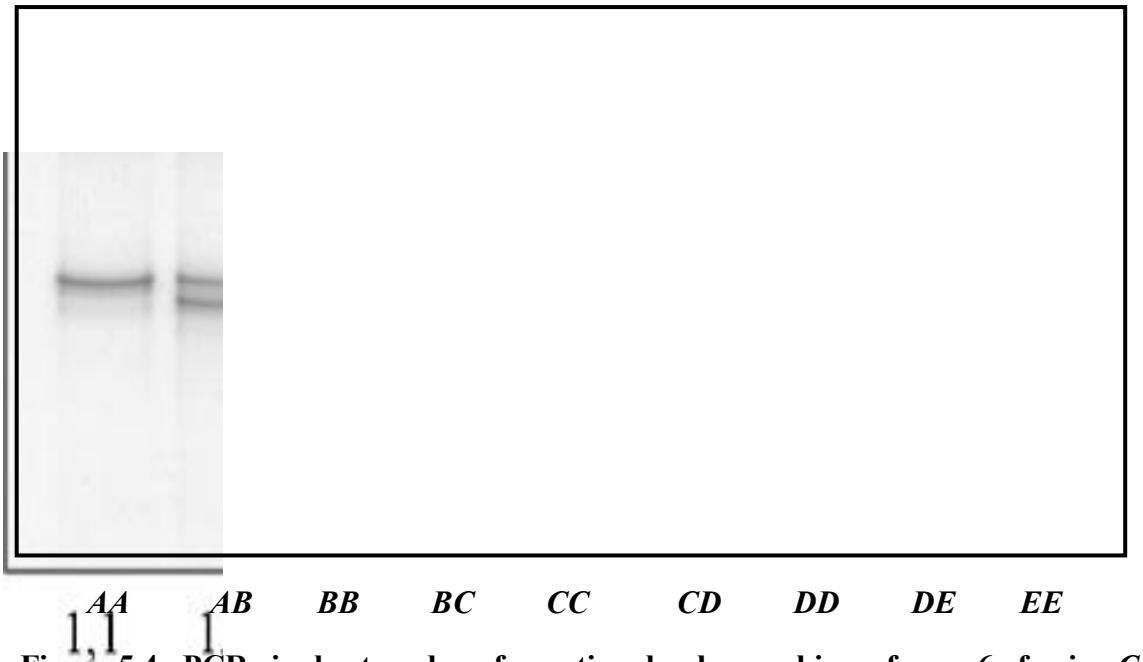


Figure 5.4. PCR-single-strand conformational polymorphism of exon 6 of ovine *CAST*. Representative sheep for the five unique SSCP patterns corresponding to known five sequences (*A*-*E*)

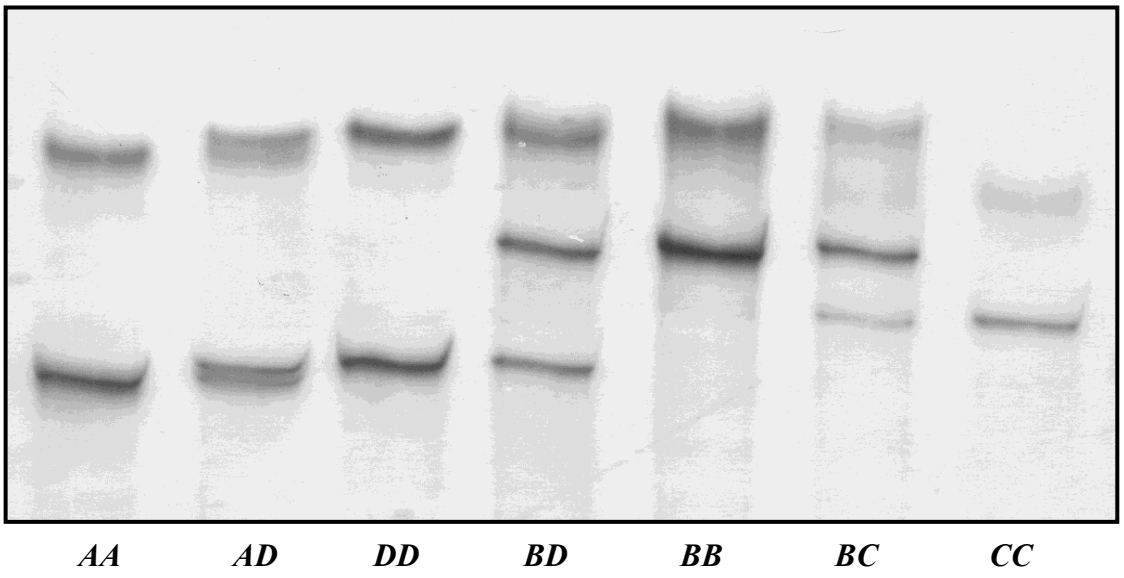


Figure 5.5. PCR-single-strand conformational polymorphism of intron 12 of ovine *CAST*. Sheep homozygous for the four unique SSCP patterns are shown plus some heterozygous sheep

5.2.1 Haplotypic diversity within ovine *CAST*

The polymorphism found in region 1 (exon 6) and 2 (intron 12) allowed the determination of *CAST* haplotypes. Nine haplotypes were identified and these results are presented in table 5.3. The haplotypes identified suggest that historically at least one intragenic recombination event has occurred in ovine *CAST*, somewhere between the two amplified regions (Figure. 5.6).

Table 5.2. Sequences of intron 12 of ovine *CAST*.

Nucleotide Position[a]	*A*	*B*	*C*	*D*
78	T	G	—	—
114	A	—	G	—
200	G	A	—	—
233	G	—	A	—
266	G	A	—	—
271	G	—	T	—
287	C	T	—	—
357	A	G	—	—
440	C	T	—	—
453	•	•	•	T

[a] Position is given relative to the ovine *CAST* reference sequences (GenBank accession numbers AF016006-AF016008 for sequences *A*-*C*, respectively), with the new sequence *D* (GenBank accession number EF669476). Bars represent nucleotides identical to the nucleotides of sequence *A* and dots show the absence of nucleotides in this position of the *A*, *B*, *C* sequences compared with sequence *D*

Table 5.3. Nine different haplotypes spanning region 1 and region 2 of ovine *CAST*.

Haplotype	Region 1 ——— Region 2 (intron 5-exon 6) (intron 12)
1	*A* ——— *A*
2	*A* ——— *B*
3	*A* ——— *C*
4	*B* ——— *A*
5	*B* ——— *C*
6	*C* ——— *B*
7	*C* ——— *D*
8	*D* ——— *A*
9	*E* ——— *C*

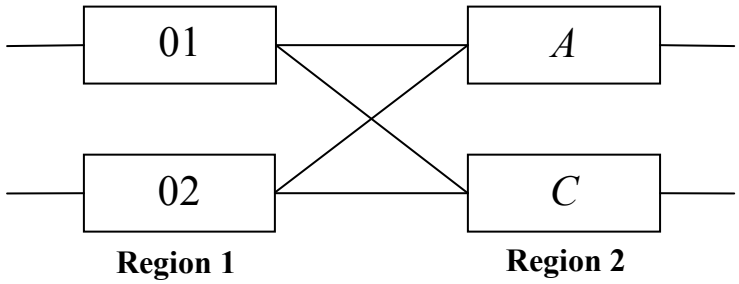


Figure 5.6. Diagram showing recombination between region 1 (part of intron 5-entire exon 6) and 2 (part of intron 12) revealed by *CAST* haplotype analysis.

5.2.2 Association of ovine *CAST* variation in exon 6 and birth weight and growth rate to weaning

A significant positive correlation between birth weight and growth rate to weaning existed (Pearson correlation coefficient, r = 0.306; $P \leq 0.001$). However, birth weight was a poor predictor of growth rate to weaning with an accuracy of 9.4% ($r^2 = 0.094$). For each *CAST* sequence, an ANOVA was performed using the significance level of $\alpha = 0.05$ to assess the effect of sequence presence (or absence), sire and birth rank (as well as the interactions of these factors) on birth weight and growth rate.

The presence of the *CAST A* had a significant effect on birth weight, but was not found to have a significant effect on growth rate to weaning (Table 5.4). A significant interaction between birth rank and *CAST A* ($P = 0.005$) was also detected for birth weight. When the data was split by birth rank and the analysis repeated, the significant effect of *CAST A* on birth weight, was only observed in single born lambs ($P = 0.007$) with those lambs possessing *CAST A* having a mean birth weight of 6.890 ± 0.183 kg (n = 27), whereas lambs that did not possess the sequence had a mean birth weight of 6.328 ± 0.130 kg (n = 70). No effect was observed in the multiple born lambs ($P = 0.597$).

The presence of *CAST B* had no significant effect on birth weight or growth rate (Table 5.4). However, for growth rate, an interaction between birth rank and *CAST B* was observed ($P = 0.038$). When the data were split by birth rank and the analysis repeated, no significant effect of the presence of *CAST B* on growth rate to weaning was detected in either singles ($P = 0.077$) or multiples ($P = 0.186$).

The presence of *CAST C* had a significant effect on birth weight, but did not have a significant effect on growth rate (Table 5.4). A significant interaction between birth rank and the presence of *CAST C* on growth rate was observed ($P = 0.034$). When these data were split by birth rank and the analysis repeated, the presence of *CAST C* had a significant effect on single born lambs ($P = 0.034$), with those lambs possessing the sequence having a mean growth rate of 336.032 ± 11.092 g/day (n = 16), whereas lambs that did not possess the *CAST C* sequence had a mean growth rate of 319.544 ± 6.167 g/day (n = 78). No effect of the presence of *CAST C* on growth was detected in multiples ($P = 0.338$).

Analyses of variance ($\alpha = 0.05$) were also used to assess the effect of sequence copy number (0, 1 or 2 copies), sire and birth rank (as well as the interactions of these factors) on birth weight and growth rate. Unfortunately, limited homozygosity (*AA*, n = 3; *BB*, n = 261; *CC*, n = 3) in the lamb population precluded analyses for *CAST A* and *CAST C*. The copy number of *CAST B* sequences was not found to effect either birth weight or growth rate to weaning ($P = 0.287$ and $P = 0.664$, respectively). A full factorial model was employed to test the effect of genotype of ovine *CAST* on birth weight. A significant difference was observed between the mean birth weight of those animals with the *AB* or *BB* genotypes when compared to those with the *BC* genotype (LSD, $P = 0.001$ and $P = 0.005$, respectively). The mean birth weight of those animals with the *BC CAST* genotype was lighter (*AB* 6.161 ± 0.100, *BB* 6.017 ± 0.076, *BC* 5.591 ± 0.130, $P = 0.004$, Table 5.4).

In general, birth weight has a positive phenotypic correlation with growth rate. Therefore, a Pearson Correlation was performed to investigate the relationship between birth weight and growth rate up until weaning. However, the CAST birth weight effect appears to be independent of growth grate as the presence of the *CAST A*, *B* and *C* sequences did not have a significant effect on growth rate ($P = 0.774$, $P = 0.384$ and $P = 0.229$), respectively. This result suggests that those ovine *CAST* sequences associated with heavier and lighter mean birth weight, have no effect on growth rate.

Table 5.4. Associations between variation in ovine *CAST* exon 6 and birth weight and growth rate, estimated by analyses of variance.

CAST	Status	n	Birth weight (kg)	Growth rate (g)
A	Present	137	**6.08 ± 0.09**	291.09 ± 5.29
	Absent	325	**5.91 ± 0.07**	299.13 ± 3.83
			(*P* = 0.019)	(*P* = 0.774)
B	Present	443	6.02 ± 0.06	297.53 ± 3.36
	Absent	19	5.57 ± 0.22	285.67 ± 11.88
			(*P* = 0.143)	(*P* = 0.384)
C	Present	77	**5.53 ± 0.12**	292.74 ± 6.92
	Absent	385	**6.14 ± 0.07**	296.23 ± 3.86
			(*P* ≤ 0.001)	(*P* = 0.229)
AB		121	**6.16 ± 0.10**	
BB		263	**6.08 ± 0.08**	
BC		61	**5.59 ± 0.13**	
			(P = 0.004)	

5.2.3 No association between variation in ovine *CAST* exon 6 and either longevity or fecundity in sheep

Genotypes *AA* (n = 320, 18.6%), *AB* (n = 594, 34.6%), *AC* (n = 83, 4.8%), *AD* (n = 5, 0.3%), *BB* (n = 556, 32.4%), *BC* (n = 142, 8.3%), *BD* (n = 4, 0.2%), *CC* (n = 13, 0.8%), *CD* (n = 1, 0.1%) and *BE* (n = 1, 0.1%) were observed, with sequence frequencies of *A* (38.4%), *B* (53.8%), C (7.4%) and *D* (0.4%) respectively. The rare *E* sequences were not included in further statistical analysis. Each *CAST* sequence was tested for its effect on both longevity and fecundity, using a significance level of $\alpha = 0.05$. A univariate GLMM (general linear mixed model) was utilized to analyse the effect of the presence or absence of each variant sequence on the traits (Table 5.5 and Table 5.6). The effect of breed and flock was corrected for as a direct effect. There was no significant association between either longevity or fecundity and variation in the *CAST* gene when adjusted for major effects (Table 5.5 and Table 5.6).

Table 5.5. Association between ovine *CAST* variation in exon 6 and variation in longevity by unvariate analyses.

CAST	Status	n	Age (Unadjusted means)	Age (Breed adjusted)	Age (Flock adjusted)
A	Present	1007	5.23 ± 3.31	5.67 ± 0.10	5.11 ± 0.10
	Absent	719	5.54 ± 3.22	5.57 ± 0.13	5.09 ± 0.13
			(*P* = 0.052)	(*P* = 0.535)	(*P* = 0.867)
B	Present	1302	5.37 ± 3.21	5.59 ± 0.10	5.08 ± 0.09
	Absent	424	5.35 ± 3.47	5.74 ± 0.16	5.15 ± 0.16
			(*P* = 0.924)	(*P* = 0.410)	(*P* = 0.730)
C	Present	239	6.17 ± 3.18	5.94 ± 0.20	5.35 ± 0.21
	Absent	1487	5.23 ± 3.37	5.57 ± 0.09	5.065 ± 0.081
			(*P* = 0.001)	(*P* = 0.098)	(*P* = 0.203)
D	Present	10	5.60 ± 3.89	5.89 ± 0.98	5.197 ± 0.10
	Absent	1716	5.36 ± 3.27	5.62 ± 0.08	5.101 ± 0.08
			(*P* = 0.818)	(*P* = 0.785)	(*P* = 0.924)

Table 5.6. Association between ovine *CAST* variation in exon 6 and variation in fecundity by unvariate analyses.

CAST	Status	n	Average lambs/year (Unadjusted means)	Average lambs/year (Breed adjusted)	Average lambs/year (Flock adjusted)
A	Present	402	1.71 ± 0.46	1.72 ± 0.02	1.77 ± 0.02
	Absent	256	1.86 ± 0.47	1.72 ± 0.03	1.78 ± 0.02
			(*P* = 0.001)	(*P* = 0.962)	(*P* = 0.808)
B	Present	494	1.81 ± 0.47	1.71 ± 0.02	1.77 ± 0.02
	Absent	164	1.65 ± 0.47	1.74 ± 0.03	1.79 ± 0.03
			(*P* = 0.001)	(*P* = 0.305)	(*P* = 0.546)
C	Present	73	1.87 ± 0.42	1.79 ± 0.04	1.82 ± 0.04
	Absent	585	1.76 ± 0.48	1.71 ± 0.02	1.77 ± 0.01
			(*P* = 0.063)	(*P* = 0.105)	(*P* = 0.189)
D	Present	4	1.49 ± 0.29	1.93 ± 0.19	2.01 ± 0.17
	Absent	654	1.77 ± 0.47	1.72 ± 0.01	1.77 ± 0.01
			(*P* = 0.229)	(*P* = 0.263)	(*P* = 0.153)

5.3 Discussion

There are a number of studies reporting variation in *CAST* exon 6 (H. Zhou et al., 2007a) and intron 12 (Palmer et al., 1998; Palmer et al., 2000; Roberts et al., 1996). In this chapter, using a large number of sheep, I investigated these regions to identify if more variation exists and if this variation is associated with various production traits including longevity and fecundity in sheep.

Previously, three sequences had been described at intron 12 of ovine *CAST* (Roberts et al., 1996), but the PCR product that was used to reveal these was too large for optimal use in PCR-SSCP analysis. I therefore designed new primers within this region to amplify a smaller fragment, that included all known polymorphic sites and which in turn resulted in the detection of a four sequences.

All the previously described nucleotide variation in exon 6 was found in the sheep studied, along with a new intron 12 sequence. An analysis of the extended haplotypes through the exon 6 to intron 12 region revealed nine haplotypes and with evidence of recombination between the two regions that were typed. At least one recombination event had occurred historically in the region between exon 6 and intron 12 (Figure 5.6).

As no complete ovine CAST gene sequence is yet available, the bovine CAST gene sequence was searched for potential sequence motifs associated with DNA recombination. Multiple *chi* sequences (5'-GCTGGTGG-3') were presented in intron 4, intron 7 and intron 17 of bovine *CAST*. In addition, *chi*-like sequences (GCTGGG) are found in a few intron regions of the bovine gene and the reported ovine mRNA sequences. Finally an 8-mer perfect inverted repeat sequence (CAATATGT/ACATATTG) was found between nucleotides -40 and -49 upstream of the ovine *CAST* exon 6 locus. Inverted repeat (or palindromic) sequences have been reported to be associated with break points during recombination events. The presence of *chi* sequences (5'-GCTGGTGG-3') (Giordano et al., 1997), *chi*-like sequences (GCTGGG) (Matsuno et al., 1992) and palindromic sequences (Krawinkel et al., 1986) has been reported to be important for gene conversion, and so it might be hypothesised that this is generating new haplotypes at the ovine CAST gene. Intragenic recombination is not uncommon, and recently there have been reports of it occurring in the Norwegian goat casein genes (Hayes et al., 2006) and European rabbit casein *CSN3* (Carneiro & Ferrand, 2007).

Several studies have reported genetic variation in bovine *CAST*, including coding region variation (Chung et al., 1999) and non-coding region variation (Juszczuk-Kubiak et al., 2008; Schenkel et al., 2006).

Extended haplotypes of *CAST* have not been reported in cattle and accordingly, undetected variation potentially including intragenic recombination may well occur in this species. This may weaken the utility of the current bovine *CAST* gene-markers for meat tenderness, which are based around the testing of single nucleotide variation in *CAST*. For example, the Igenity *Tender*-GENE marker panel consists of two μ-calpain SNPs and a *CAST* SNP known as *UoG-CAST* (Schenkel et al., 2006). The *UoG-CAST* marker is a G/C nucleotide substitution in the intronic sequence between exon 5 and 6 of *CAST* (base 282 of accession # AY008267). In contrast, a A2959G SNP in the 3' un-translated region (UTR) is used in the GeneSTAR Tenderness test panel (Genetic Solutions Pty. Ltd., Albion, Australia).

The finding of nine extended haplotypes in sheep suggests that cattle may also be more polymorphic at *CAST* than has been reported. This contention would be supported by the four haplotypes described in a reasonably small (n = 570) sample of pigs (Barendse, 2002). The failure to recognise more extensive or complex polymorphism in bovine *CAST* and the possibility of intragenic recombination may also explain why a recent large-scale study of Argentine beef cattle failed to confirm the value of the commercial gene-marker tests (Corva et al., 2007). This study utilised the GeneSTAR Tenderness test. If the single nucleotide substitutions typed in this commercial test was associated with variation elsewhere in the gene that was not consistent with what Barendse et al. (2002) had described previously and that also affected meat tenderness in a different way, then Corva et al. (2007) results could be explained. In effect a single nucleotide substitution would not be revealing the true complexity of the genome or its potential phenotypic effects.

Haplotypic diversity in ovine *CAST* seems to be high with evidence of recombined haplotypes. This might be important in generating even greater genetic diversity. It is however conceivable that further genetic variation exists, especially when more sheep from different breeds are analysed and as genotyping methods are developed that enable variation across the extended CAST gene region to be easily assessed. An understanding of the nature and extent of *CAST* genetic diversity across a large number of breeds should enable more comprehensive study of association between *CAST* and various phenotypes to be undertaken.

In the association study reported here a large number of sheep of known phenotype were studied. Phenotypic measures included birth weight, growth rate, longevity and fecundity. Exon 6 encoding 114 amino acids is the largest exon in the L domain (Table 5.1). The L domain has no known calpain inhibitory function, but it is known to be responsible for binding to biological membranes at the hydrophobic phospholipids (Mellgren et al., 1989). In addition, Melloni et al. (2006) have shown that the L domain binds to the catalytic domain II of calpain and that calpain undergoes a conformational change, increasing its ability to act as a protease at physiological levels of Ca^{2+} ions.

Barendse et al. (2007) have shown that nucleotide substitutions found in bovine *CAST* may have a role in affecting the activation of calpain by changing its hydropathy. They have assessed several non-synonymous amino acid substitutions, which are located in the L domain, inhibitory domains and the 3' UTR region and calculated the change in hydropathy (Kyte & Doolittle, 1982). Amino acid substitutions *CAST:c.155C>T* (Pro52Leu) and *CAST:c.143A>G* (Ser48Gly) located in the second exon of the L domain (NM_174003.2), change bovine CAST hydropathy. Pro52Leu has the largest hydropathy change from -1.6 to +3.8, while Ser48Gly changes hydropathy from -0.4 to -0.8. Pro52Leu was also associated with beef tenderness traits in the study (Barendse et al., 2007). This suggests that the increased hydropathy is associated with CAST binding activity.

Among the *CAST* sequences (*A* to *E*) reported at exon 6 (H. Zhou et al., 2007a), *CAST C* would create a non-synonymous substitution at position 134 (Gln to Leu) of the peptide. Interestingly, this amino acid substitution changes hydropathy from -3.5 to +3.8. This change is even greater than the substitution of Pro52Leu in cattle. It could therefore influence the binding of CAST to cell membranes and in the case of *CAST C*, the hydropathy change would seem to suggest a large increase in membrane-binding activity.

It has been suggested that one role of CAST in skeletal muscle growth in cattle (Chung et al., 2001), pig (Kurly et al., 2003) and sheep (Nassiry et al., 2006) may contribute to maintaining body weight in livestock. In sheep, Sumantri et al. (2008) have reported that polymorphism in *CAST* is associated with body weight. In addition, other studies have reported that genetic variation of ovine *CAPN 3* (Chung et al., 2007) and porcine *CAST* (Choi et al., 2006) are associated with birth weight and body weight in sheep and pigs respectively.

Calpain 3 (CAPN3) is a muscle-specific calpain species and has special function in maintaining muscle cells. The ubiquitous calpains (μ-calpain and m-calpain), calpain 3 and

CAST seem to have essential but distinct roles during the onset of myogenesis. *CAST* is therefore an excellent candidate gene for controlling growth in livestock.

Birth weight in sheep is one of the greatest influences upon lamb mortality due to starvation and cold exposure (Gudex et al., 2005). Lamb survival is a critical issue in attempting to increase lambing percentage, which is a major contributor to higher profits on New Zealand sheep farms. In the sheep industry, many sheep breeders and farmers have selected and bred ewes for increased fecundity. The increasing fecundity in modern sheep breeds has led to higher numbers of twins and triplets in the sheep industry. As a result, the increased proportion of ewes having triplets is of concern to farmers and to industry as lamb death is higher in multiple-born lambs than single born lambs (Everett-Hincks et al., 2007; Everett-Hincks et al., 2005b; Kerslake et al., 2005). Lambs from multiple births are typically smaller at birth.

There is a clear relationship between lamb birth weight and the survival rate of lambs. This indicates that increasing birth weight could be an advantage to the survival of twin and triple lambs. On the other hand, lambs with heavier birth weights have been reported to be the predominant factor leading to lambing problems or dystocia in sheep (Fogarty, 1992). Traditionally, heavier lambs were thought to contribute to the incidence of dystocia in sheep (Alexander, 1984), especially with single lambs (Fogarty, 1992) and that this leads to the death of many lambs and ewes. In contrast, it has recently been suggested that dystocia plays a significant role in triplet-born lamb deaths (Everett-Hincks & Dodds, 2008; Kerslake et al., 2005) Everett-Hincks et al, 2008), which collectively would suggest that overly light and overly heavy lambs would cause issues with lamb survival. Accordingly, although increasing birth weight might improve lamb survival, it needs to be balanced against possible increases in dystocia. Equally while increasing fecundity may improve productivity, it might also lead to increased number of dystocia prone triplets, suggesting a "balance" is needed between lamb size and fecundity.

Ovine *CAST* variation in exon 6 had a significant effect on birth weight in single lambs, but not on growth rate in sheep (Table 5.4). The presence of *CAST A* was associated with increased average birth weight (6.084 ± 0.093, $P = 0.019$) while the presence of *CAST C* was associated with decreased average birth weight (5.529 ± 0.122, $P \leq 0.001$). This suggests that CAST is a regulator of birth weight in sheep and it may enable better management of optimum birth weight.

There was no significant association between either longevity or fecundity and variation in ovine *CAST* exon 6 in any of the analyses (Table 5.5 and 5.6). This would suggest that either 1) the sequences described in H. Zhou et al. (2007) may not affect longevity or fecundity in sheep, or 2) that the variation that may affect longevity or fecundity is found in another region of the CAST gene or 3) that in sheep, unlike cattle (Garcia et al., 2006) this gene does not affect these traits.

Other studies have not found genetic associations with longevity and fertility in different livestock. It might therefore be that the *CAST* variation in cattle may affect some other trait or traits that would lead indirectly to a cow being culled. In dairy cattle, the major "risk factors" for being culled are reduced milk production, increased lameness and mastitis susceptibility. If *CAST* affects any of these traits directly, then indirectly it may also affect longevity and fertility in dairy cattle. In this respect it might be interesting to look at the effect of ovine *CAST* variation on longevity and fertility in milking sheep.

Chapter 6
A role for TLR4 in longevity and fecundity?

Toll-like receptors (TLRs) are highly conserved trans-membrane proteins that play an important role in the recognition and elimination of microbial pathogens (Uematsu & Akira, 2006). Recognition of their ligands leads to stimulation of a series of signalling pathways resulting in acute host responses (Blander, 2008; Netea et al., 2008). These signalling pathways play an important role in immune response and inflammation, and are critical for host survival.

One of the best understood TLR members is TLR4, identified at the genetic level as the first human homologue of the *Drosophila* Toll gene (Hashimoto et al., 1988; Medzhitov et al., 1997). TLR4 is expressed on different immune cells, including classic antigen-presenting cells (dendritic cells, monocytes and macrophages), as well as B and T cells. This suggests that the receptor has a primary function in both innate and acquired immunity (Blander, 2008; Netea et al., 2008; Richards et al., 2008). Different tissues can also respond to inflammatory stimuli via TLR4, with its expression being observed on epithelial cells, including cells in the skin, respiratory, intestinal and genitourinary tracts, and on endothelial and smooth muscle cells (Lu et al., 2008).

Structurally, TLR4 consists of three domains: an extracellular leucine-rich repeat (LRR) domain, a trans-membrane domain and an intracellular Toll-interleukin-1 receptor (TIR) domain (Figure 6.1). The extracellular LRR domain is involved in recognition of the lipopolysaccharide (LPS) of Gram-negative bacteria, the prototypic TLR4 ligand. Other known exogenous TLR4 ligands are the fusion protein of respiratory syncytial virus and the envelope protein of mouse mammary tumour virus (Lu et al., 2008; Uematsu & Akira, 2008). In addition, endogenous molecules can directly or indirectly interact with TLR4, such as heat-shock proteins (HSPs), hyaluronic acid, β-defensin-2, oxidized-LDL, fibronectin, and amyloid peptide (Lu et al., 2008; Tsan & Gao, 2004). Thus, TLR4 can be activated by a variety of exogenous ligands, as well as endogenous molecules.

Activation of TLR4 mediates the activation of transcription factors, such as NF-*k*B, and consequently the production of different inflammatory mediators including various cytokines and chemokines (Lu et al., 2008; Tsan & Gao, 2004). TLR4 activation also induces the expression of co-stimulatory molecules and the major histocompatibility complex class II

antigens, molecules which contribute to the establishment of an acquired immune response (Blander, 2008; Miyake, 2007; Netea et al., 2008; Richards et al., 2008; Zeytun et al., 2007).

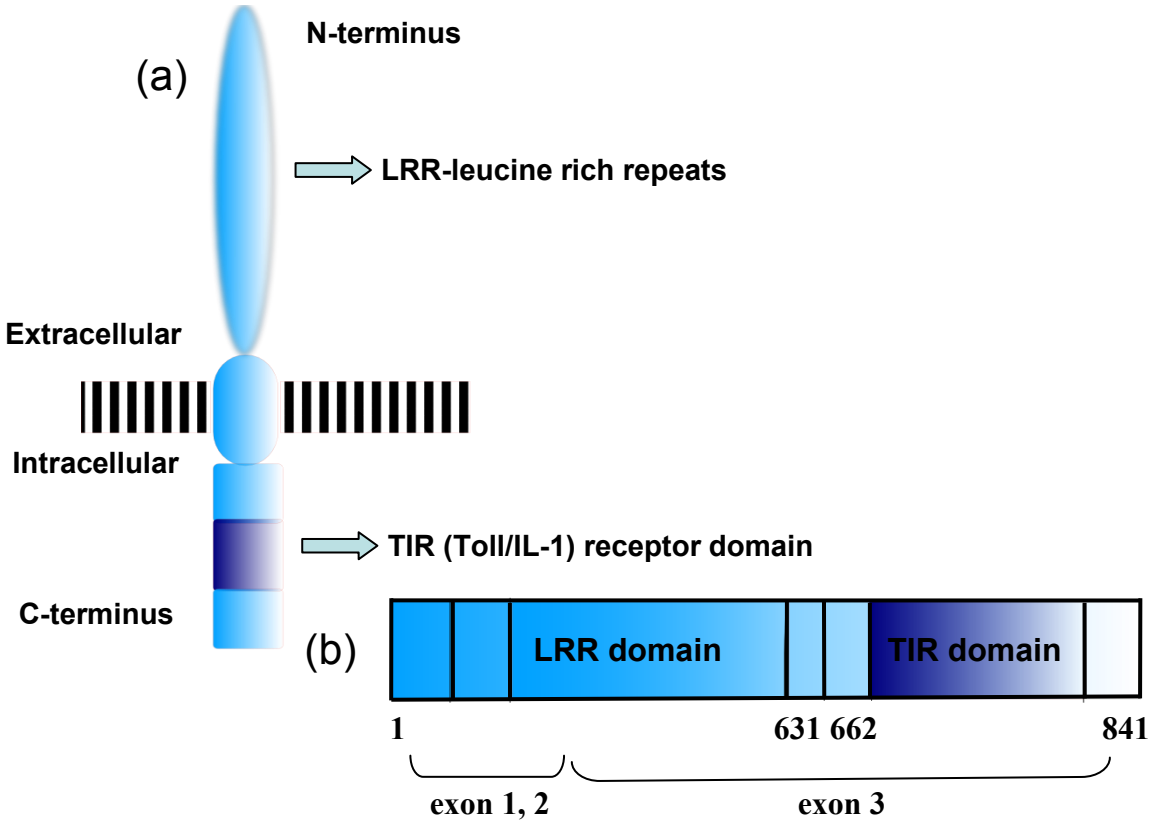


Figure 6.1. The structure of bovine TLR4. (a) Trans-membrance proteins with an extracellular leucine-rich repeat (LRR) domain and a cytoplasmic tail, referred to as the Toll-interleukin 1 receptor (TIR) domain. (b) Structural domain organization of bovine *TLR4*: signal sequence (1-23), putative co-receptor-binding region 1(24-273), putative ligand-binding region (274-368), putative co-receptor-binding receptor 2 (369-632), transmembrane region (633-653), proximal cytoplasmic region (654-672), TIR domain (673-819) and distal cytoplasmic region (820-839) (Takeda et al., 2003; White et al., 2003)

- **A role for TLR4 in longevity?**

During the aging process, the function and expression of TLR4 may impair activation of the immune response, leading to an increasing incidence of infectious and age-related diseases. This may consequently contribute to a decrease in longevity. What-is-more, in elderly humans, many alterations in the immune system have been observed and recorded as deleterious, leading to definition of the term immunosenescence to describe this state (Ostan et al., 2008; Vasto & Caruso, 2004).

Chronic antigen loading from bacteria, viruses, fungi seems to be the major factor in immunosenescence, and it impacts by reducing the number of naïve T cells while filling the "immunological space" with expended memory cells (Wherry & Ahmed, 2004). The immune system therefore loses its ability to mount effective acquired responses, especially to new antigens. Antigenic stress can also continuously stimulate the immune system through the activation and generation of inflammatory responses. The increase in inflammation has been shown to be a factor in age-related diseases such as atherosclerosis and cardiovascular diseases, Alzheimer disease (Balistreri et al., 2009) and cancer (Grivennikov et al., 2010). These are all causes of morbidity and mortality in the elderly.

Evidence of a role for TLR4 in aging was initially derived from studies on the LPS-mediated cytokine responses in blood samples from younger and older subjects (Krabbe et al., 2004; Pedersen & Bruunsgaard, 2003; Rink et al., 1998). In these studies, TLR4 expression on neutrophils and lymphocytes was assessed in the young and the elderly subjects using a flow cytometry approach (Fulop et al., 2004; Van Duin & Shaw, 2007). Interestingly, no changes in TLR4 expression on neutrophils was observed with aging, but increased TLR4 expression was found in specific membrane-signalling micro-domains (called lipid rafts) and non-raft membranes in older subjects without LPS stimulation, when compared to the neutrophils of the younger subjects (Fulop et al., 2004).

It has also been demonstrated that aging of the brain is associated with regulation of TLR4 expression. Its expression is up-regulated in mouse brains as age increased (Huang et al., 2008). The increased TLR4 expression in the brain may contribute to increased chronic inflammation, which is associated with the onset of age-related neurodegenerative diseases. Thus, longevity might be affected by things that affect the expression of TLR4.

- **A role for TLR4 in fecundity?**

TLR4 is involved in innate immunity in both the female and male reproductive systems and plays an important role in eliminating pathogens in the reproductive cells (Girling & Hedger, 2007; Herath et al., 2007; Shimada et al., 2008). Interactions between the innate immune system and reproductive system have important consequences for fecundity and reproductive health in general. In the female, TLR4 has been implicated in critical aspects of ovarian, endometrial and placental function, as well as in ovarian cancer and pelvic inflammatory disease (Fazeli et al., 2005; Horne et al., 2008). In the male, TLR4 appear to play a role in the control of testicular steroidogenesis and spermatogenesis in disease, and

potentially during normal function as well (Palladino et al., 2007; Rodrigues et al., 2008). Recent studies have suggested a role for TLR4 in prostate cancer (Cheng et al., 2007).

There is increasing evidence for the involvement of TLR4 in fertilisation, and the physiology of reproduction. In female mouse, cow and human ovaries, TLR4 is expressed and induced in granulose and cumulus cells (Salustri et al., 2004; Varani & Matzuk, 2002). During fertilisation, sperm cells induce cumulus cells to release specific cytokines and chemokines. Shimada et al. (2008) have shown that this event is dramatically reduced in the presence of TLR4 blocking antibodies and suggest that the expression of TLR4 may be important in regulating the interaction between sperm cells and cumulus cell-oocyte complexes.

TLR4 expression has also been reported in uterine (Soboll et al., 2006) and oviductal tissue and it is reported to affect pre-term labour (Girling & Hedger, 2007). TLR4 may also respond to factors in addition to sperm that enter the reproductive tract, including bacteria (Girling & Hedger, 2007). Based on these observations, the role of TLR4 in the reproduction system has received significant attention from reproduction biologists, and the possibility that it directly affects fecundity in sheep is certainly worthy of investigation.

- **Variation in *TLR4***

A cDNA sequence for bovine *TLR4* has been reported, with 72% and 65% amino acid similarities to human and mouse TLR4, respectively (Werling & Jungi, 2003). In addition, table 6.1 shows that bovine, porcine, human, murine and avian *TLR4* share the same genomic structure. This suggests that the TLR4 gene is conserved, with a similar genetic structure among various species, including sheep.

Table 6.1. Comparison of the size in bp of various parts of the human, pig, cattle, mouse and chicken TLR4 gene. Adapted from (Leveque et al., 2003)

	Human	*Pig*	*Cow*	*Mouse*	*Chicken*
Exon1-cds	**93**[a]	**93**	**93**	**93**	105
Intron 1	3997	3866	4547	5972	934
Exon 2	**167**[b]	**167**	**167**	**167**	**167**
Intron 2	3659	2582	2749	5168	984
Exon 3-cds	2260	2265	2266	2251	2260

[a]-Same size found in sheep, pygmy chimpanzee and live baboon
[b]-Same size found in sheep, gorilla, pygmy chimpanzee and olive baboon

Sequencing of *TLR4* in individual mammals has identified, intra-species genetic variation in the LRR ligand-recognition domain (Smirnova et al., 2000), while the TIR domain was highly conserved across species. The diversity in the LRR domain seems to accommodate recognition of different pathogen-associated molecular patterns (PAMPs) in different species, and this is hypothesised to be the result of evolutionary pressure induced by pathogens on the host (Mushegian & Medzhitov, 2001).

The LRR domain of TLR4 contains LRRs of different size and abundance. These LRRs are protected by special LRR-N terminal end and LRR-C-terminal end motifs. The defining feature of an LRR repeat is the highly conserved 11-residue hallmark sequence LxxLxLxxNxL (with 'x' being any amino acid). Other hydrophobic residues can substitute the leucine residues at the consensus positions (Matsushima et al., 2007). Within each LRR repeat, the conserved residues provide a rigid structural framework, whereases variable residues are available for interaction with ligands. This interaction is further supported by insertions into the highly conserved 11 amino acid segment (Bell et al., 2003; Kajava et al., 1995; Kobe & Deisenhofer, 1995) (Table 6.2).

There is evidence that variation in the LRRs of *TLR4* is associated with altered immune responses to pathogens and results in variation in disease susceptibility. Smirnova et al. (2001) have sequenced the entire TLR4 gene of 348 humans of different ethnic background and reported on the presence of SNPs in both the extracellular and cytoplasmic domains. They found Asp299Gly and Thr399Ile in the TLR4 extracellular domain in Caucasian populations. These occur in the LRR region of LRR11 and LRR 15, respectively (Table 6.2). These substitutions have recently been shown to reduce TLR signalling (Arbour et al., 2000) and change the ligand-binding properties of the receptor (Rallabhandi et al., 2006), and this may negatively influence immune responses. In this respect, they might be considered to be mutations. In addition, Ohara et al. (2006) reported that one mutation, Thr135Ala in LRR5, was associated with gastric carcinoma. Variation in *TLR4* could therefore become important in understanding the role of genetically encoded control of immunity, which might affect both longevity and fecundity.

Mounting evidence for associations between *TLR4* variation and immunological-related traits in mammals, has important implications for livestock. TLR4, is a major sensor of infections by Gram-negative bacteria, and is a strong candidate gene for variation in response to a number of commercially important animal diseases related to impaired respiratory

immune protection (Chapes et al., 2001; Hawn et al., 2005) and intestinal infections (Eckmann, 2006; Fukata et al., 2005).

Table 6.2. The twenty-three reported LRRs in human TLR4.

TLR4 LRR	Position [a]	Leucine-rich (LxxLxLxxNxL) repeat	Mutation
LRR1	35-55	NITYQCMELNF YKIPDNLPFS	
LRR2	56-79	TKNLDLSFNPL RHLGSYSFFSFPE	
LRR3	80-103	LQVLDLSRCEI QTIEDGAYQSLSH	
LRR4	104-127	LSTLILTGNPI QSLALGAFSGLSS	
LRR5	128-151	LQKLVAVE**T**NL ASLENFPIGHLKT	T135A
LRR6	152-176	LKELNVAHNLI QSFKLPEYFSNLTN	
LRR7	177-204	LEHLDLSSNKI QSIYCTDLRVLHQMPLL	
LRR8	205-227	NLSLDLSLNPM NFIQPGAFKEIR	
LRR9	228-254	LHKLTLRNNFD SLNVMKTCIQGLAGLE	
LRR10	255-284	VHRLVLGEFRN EGNLEKFDKSALEGLCNLT	
LRR11	285-309	IEEFRLAYLDY YLD**D**IIDLFNCLTN	D299G
LRR12	310-331	VSSFSLVSVTI ERVKEFSYNFG	
LRR13	332-352	WQHLELVNCKF GQFPTLKLKS	
LRR14	353-374	LKRLTFTSNKG GNAFSEVDLPS	
LRR15	375-400	LEFLDLSRNGL SFKGCCSQSDFGT**T**S	T399I
LRR16	401-423	LKYLDLSFNGV ITMSSNFLGLEQ	
LRR17	424-448	LEHLEFQHSNL KQMSEFSVFLSLRN	
LRR18	449-472	LIYLDISHTHT RVAFNGIFNGLSS	
LRR19	473-497	LEVLKMAGNSF QENFLPDIFTELRN	
LRR20	498-521	LTFLDLSQCQL EQLSPTAFNSLSS	
LRR21	522-545	LQVLNMSHNNF FSLDTFPYKCLNS	
LRR22	546-570	LQVLDYSLNHI MTSKKQELQHFPSS	
LRR23	571-595	LAFLNLTQNDF ACTCEHQSFLQWIK	

[a] Amino acid position relative to the human *TLR4* reference sequence (GenBank accession number AAF05316). Some reported missense mutations are highlighted in red.

In sheep, 14 SNPs have been detected in exon 3 of ovine *TLR4* which contains a putative ligand binding region, while only seven SNPs are found in the corresponding regions of bovine *TLR4* (H. Zhou et al., 2007b). This suggests that sheep are more diverse than cattle at this locus, although the reason for this is not known.

The effect of genetic variation in exon 3 of ovine *TLR4* has not been studied and nothing is known of if effects on longevity and fecundity. Exon 3 encodes the LRR, and in this chapter, using a large number of sheep, I investigated this region to identify if more variation exists, and if this variation is associated with ovine longevity and fecundity.

6.1 Materials and Methods

6.1.1 Sheep investigated to define genetic variation in exon 3 of *TLR4*

In total 1670 Merino, Corriedale, NZ Romney, Poll Dorset, and other NZ cross-bred sheep were investigated in this study. These sheep were selected from different stud farms in New Zealand.

6.1.2 Sheep and data collection for assessing longevity and fecundity

Sheep blood samples with sheep longevity and fecundity data were used (See chapter 2.2 for sheep studied and the collection of longevity and fecundity data) to assess the effect of genetic variation of *TLR4* on sheep longevity and fecundity.

6.1.3 DNA extraction from blood on FTA cards

(See chapter 3.1.3)

6.1.4 PCR primer design and synthesis

To amplify fragments of suitable size for SSCP analysis, PCR primers were designed based on the published ovine sequences (H. Zhou et al., 2007b) (Figure 6.2), to amplify a 256 bp fragment within exon 3 of the ovine *TLR4*. These were *TLR4*-up2 (5'-TCTTTAGGAAGTCTACAAGC-3') and *TLR4*-dn2 (5'-AATGCTTCAGGTTGGTTGTC-3'). The primers were synthesised by Integrated DNA Technologies (Coralville, IA, USA).

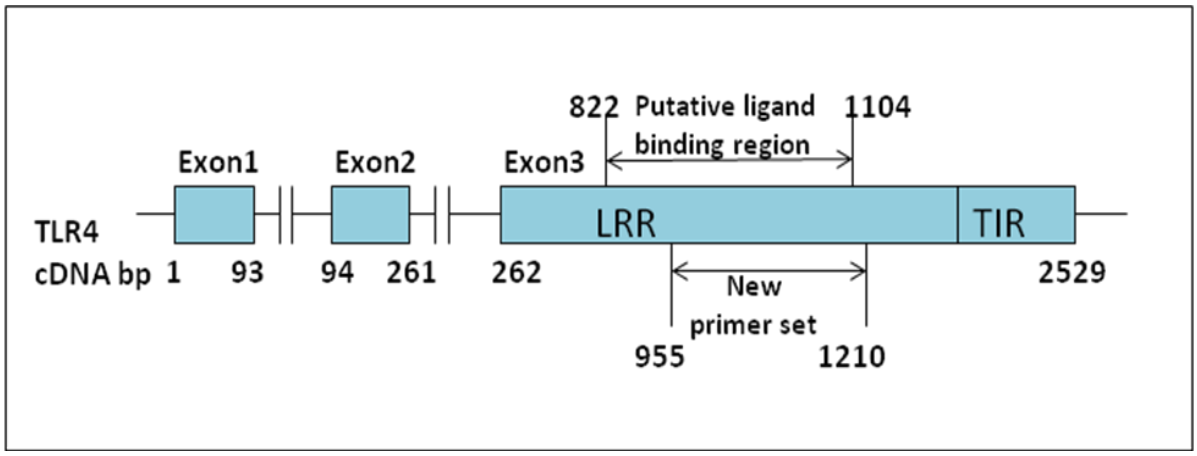


Figure 6.2. The location of PCR primers in ovine *TLR4*. The position of TLR4 cDNA is related to the bovine *TLR4* sequences (GenBank accession no. AY277040). LRR=leucine rich region; TIR=Toll-interleukin 1 receptor domain

6.1.5 PCR-SSCP analysis

Amplifications were performed in a 20 µL reaction containing the genomic DNA on a 1.2 mm punch of FTA card, 0.25 µM of each primer, 150 µM dNTPs (Eppendorf, Hamburg, Germany), 0.5 U *Taq* DNA polymerase, and 1x the reaction buffer supplied with the enzyme (containing 1.5 mM $MgCl_2$) (Qiagen, Hilden, Germany). Amplification was carried out in an iCycler (Bio-Rad Laboratories, Hercules, CA, USA) and consisted of denaturation at 94 ºC for 2 min, followed by 35 cycles of 94 ºC for 30 s, 60 ºC for 30 s, and 72 ºC for 30 s, with a final extension step at 72 ºC for 5 min. Amplicons were visualised by electrophoresis in 1% Seakem LE agarose (Bio Whittaker Molecular Applications, Rockland, ME) gels, using 1 x TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM Na_2EDTA) containing 200 ng/mL ethidium bromide.

A 0.7 µL aliquot of each amplicon was mixed with 7 µL of loading dye (98% formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene-cyanol), and after denaturation at 95 ºC for 5 min, samples were rapidly cooled on wet ice and then loaded on 16 cm × 18 cm, 14% acrylamide:bisacrylamide (37.5:1: Bio-Rad) gels. Electrophoresis was performed using Protean II xi cells (Bio-Rad), at 300 V for 18 h at 4 ºC in 0.5 × TBE buffer. The gels were silver-stained according to the method of Sanguinetti et al. (1994).

6.1.6 DNA sequencing and sequence analysis

(See chapter 3.1.6 and 4.1.6)

6.1.7 Genotyping of the ovine *TLR4* for longevity and fecundity trait analysis

The genetic association study was carried out on 1726 ewes from seven breeds which are NZ Romney (n = 340), Corriedale (n = 318), Merino (n = 326), Polwarth (n = 129), Kelso (n = 174), Coopworth (n = 439) and selected from 36 different flocks. Overall there were nine hundred and fifty eight older ewes and seven hundred and sixty-eight young ewes (2 years old). Lambing information was available for six hundred and fifty-eight of the older ewes for assessing their fecundity. These sheep were genotyped using the above method.

6.1.8 Statistical analyses

Data was analysed using SPSS version 17 (SPSS Science Inc., Chicago, IL, USA), with a two-tailed significance level of $\alpha = 0.05$.

A general linear mixed model (GLMM) was used to assess the effects of each of the *TLR4* sequences. The effect of the presence of sequences *A*, *B*, C and *D* at *TLR4* on longevity and fecundity was assessed with this model. These analyses were run as unadjusted models and additionally, in separate models adjusted for flock and breed effects. These were included as random factors.

6.2 Results

6.2.1 Development of a PCR-SSCP technique for detecting variation in ovine *TLR4*

Amplicons of 256 bp were obtained with all the sheep DNA samples using the *TLR 4*-up2 and -dn2 primers. Upon SSCP analysis, seven patterns (*A*-*G*) where observed (Figure 6.3). Either one or two of these patterns were observed for each sheep studied, which defines homozygous and heterozygous genotypes at the ovine *TLR4* locus, respectively.

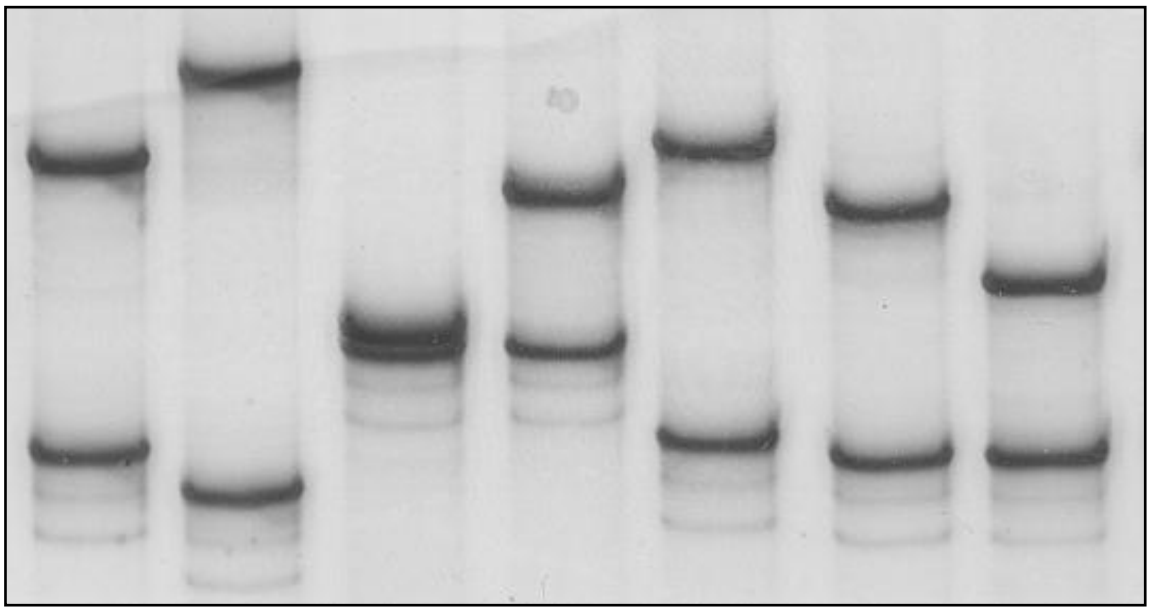


Figure 6.3. PCR-single-strand conformational polymorphism of ovine *TLR4*. Representative sheep for the seven unique SSCP patterns.

Four of the PCR-SSCP patterns (*A*-*D*) were obtained from nucleotide sequences that had been reported previously, while *E*-*G* represented three new nucleotide sequences and these were deposited into the NCBI GenBank with accession numbers EU786152-4 giving a total of seven sequences in a 256 bp fragment of this gene (Table 6.3). This variation leads to 10 different amino acid changes in the putative ligand-binding region summarised in (Table 6.3).

Table 6.3. Sequences variation in ovine *TLR4*.

SNP	Position[a]	*A*	*B*	*C*	*D*	*E*[b]	*F*[b]	*G*[b]	Amino acid substitution
T/C	955	T	T	T	T	T	C	T	Ser/Pro
T/C	1013	T	T	T	T	C	T	T	No change
G/C	1032	G	G	C	G	G	G	G	Lys/Asn
A/G	1045	A	A	A	A	A	G	A	Lys/Glu
G/A	1052	G	G	A	G	G	G	A	Arg/His
T/C	1066	T	C	T	T	T	T	T	Phe/Leu
A/G	1088	A	A	G	G	A	A	A	Asp/Gly
T/C	1091	T	T	C	C	T	T	T	Val/Ala
C/G	1097	C	C	G	G	C	G	C	Thr/Ser
C/T	1132	C	C	T	T	C	C	C	No change
G/C	1166	G	G	G	G	G	C	G	Ser/Thr
G/T	1183	G	G	T	T	G	G	G	Asp/Tyr
Sequence Frequency (n = 1670)	%	47.1	35.8	11.5	4.7	0.3	0.3	0.3	

[a] Nucleotide position relative to the bovine *TLR4* reference sequence (GenBank accession number AY297040). List accession numbers of *A-D* (DQ451551-DQ451554). [b]GenBank accession numbers for the three newly identified sequences *E-G* are EU786152 – EU786154, respectively).

Table 6.4. Position of amino acid substitutions in ovine *TLR4*.

TLR4 LRR	[a]Position	Ovine TLR4 *A* sequence Leucin-rich (LxxLxLxxNxL) repeat	TLR4 sequence	Mutation
LRR12	310-331	VSVISLLSI**S**L GSLQALLKDFR	*TLR4 F*	S319P
LRR13	332-352	WQHLEMINCDF D**K**FPAL**K**L**R**S	*TLR4 C*	K344N
			TLR4 F	K349E
			TLR4 C,G	R351H
LRR14	353-374	LKK**F**VFTDNK**D** **V**S**T**FTKTELPS	*TLR4 B*	F356L
			TLR4 C,D	D363G
			TLR4 C,D	V364A
			TLR4 C, D, F	T366S
LRR15	375-400	LQYLDLKRNHL SFK**S**CCSHT**D**FGTTN	*TLR4 F*	S389T
			TLR4 C,D	D395Y

[a] Amino acid position relative to the human *TLR4* reference sequence (GenBank accession number AAF05316). Residues of missense mutations are highlighted in red.

6.2.2 No association between variation in ovine *TLR4* and longevity, but possible association with fecundity

Table 6.5 shows the frequencies of the ovine *TLR4* genotypes in the young and old sheep. *A* and *B* are the most common sequences and account for over 80% of the total frequency.

Table 6.5. *TLR4* genotype frequencies in older and younger ewe groups.

Category		*AA*	*AB*	*AC*	*AD*	*BB*	*BC*	*BD*	*CC*	*CD*	*DD*	Total
Old	n	182	218	91	46	189	144	32	43	9	4	958
	(%)	19.0	22.8	9.5	4.8	19.7	15.0	3.3	4.5	0.9	0.4	100
Young	n	183	159	73	38	145	87	33	31	14	5	768
	(%)	23.8	20.7	9.5	4.9	18.9	11.3	4.3	4.0	1.8	0.7	100
Total	n	365	377	164	84	334	231	65	74	23	9	1726
Frequency	(%)	21.1	21.8	9.5	4.9	19.4	13.4	3.8	4.3	1.3	0.5	100

In an analysis of all of the ewes (n = 1726), sequences, *A*, *B*, *C* and *D* of ovine *TLR4* were assessed to see if they were associated with longevity and fecundity. Table 6.6 and 6.7 show the effect of longevity and fecundity for the each *TLR4* sequences in the unadjusted model, and flock and breed adjusted models.

There was no significant association between longevity and variation in the ovine *TLR4* in any adjusted models (Table 6.6).

In the fertility analysis, the unadjusted model suggests a relationship between the *A*, *B*, *C* sequences of *TLR4* and fecundity ($P = 0.001$) (Table 6.7). The presence of *TLR4 C* was associated with decreased fecundity when the model was corrected for breed (1.64 ± 0.03, $P = 0.004$). The presence of *TLR4 A* also tended to decrease fecundity when the model was corrected for flock (1.75 ± 0.02, $P = 0.066$). Other *TLR4* sequences had no association with either an increase, or decrease, of fecundity in any adjusted models.

Table 6.6. Association of variation in ovine *TLR4* with variation in longevity by univariate analysis.

Sequence	Status	n	Age (Unadjusted means)	*P* values	
A	Present	999	5.41 ± 3.34	Unadjusted	$P = 0.470$
	Absent	736	5.30 ± 3.19	Breed adjusted	$P = 0.859$
				Flock adjusted	$P = 0.928$
B	Present	1007	5.45 ± 3.22	Unadjusted	$P = 0.275$
	Absent	719	5.24 ± 3.34	Breed adjusted	$P = 0.313$
				Flock adjusted	$P = 0.275$
C	Present	492	5.39 ± 3.19	Unadjusted	$P = 0.818$
	Absent	1234	5.35 ± 3.31	Breed adjusted	$P = 0.479$
				Flock adjusted	$P = 0.298$
D	Present	181	4.71 ± 2.96	Unadjusted	$\mathbf{P = 0.004}$
	Absent	1545	5.44 ± 3.30	Breed adjusted	$P = 0.219$
				Flock adjusted	$P = 0.590$

Table 6.7. Association of variation in ovine *TLR4* with variation in fecundity by univariate analysis.

Sequence	Status	n	Average lambs/year (Unadjusted means)	Average lambs/year (Flock adjusted)	Average lambs/year (Breed adjusted)
A	Present	386	1.65 ± 0.45	1.75 ± 0.02	1.72 ± 0.02
	Absent	272	1.95 ± 0.45	1.80 ± 0.02	1.73 ± 0.03
			(*P* = 0.001)	(*P* = 0.066)	(*P* = 0.656)
B	Present	392	1.86 ± 0.46	1.79 ± 0.02	1.74 ± 0.02
	Absent	266	1.63 ± 0.46	1.75 ± 0.02	1.70 ± 0.02
			(*P* = 0.001)	(*P* = 0.143)	(*P* = 0.327)
C	Present	152	1.90 ± 0.44	1.73 ± 0.03	**1.64 ± 0.03**
	Absent	506	1.73 ± 0.48	1.78 ± 0.02	**1.75 ± 0.02**
			(*P* = 0.001)	(*P* = 0.124)	**(*P* = 0.004)**
D	Present	77	1.76 ± 0.56	1.77 ± 0.04	1.76 ± 0.04
	Absent	581	1.77 ± 0.46	1.77 ± 0.01	1.72 ± 0.02
			(*P* = 0.878)	(*P* = 0.961)	(*P* = 0.341)

6.3 Discussion

Genetic variation in *TLR4* has been associated with altered immune responses to pathogens, and variation in disease susceptibility in humans and other mammals. In this chapter, using a large number of sheep, I investigated genetic variation in ovine *TLR4* and if this variation is associated with longevity and fecundity.

Genetic variation in exon 3 of ovine *TLR4* has been described previously with four sequences identified (H. Zhou et al., 2007b). However, a simple and reliable genotyping method for ovine *TLR4* variation was required to enable the study of large numbers of sheep. In this chapter, PCR-SSCP conditions were developed to detect variation in exon 3, and so as to supersede less reliable methods including PCR-restriction fragment length polymorphism analysis (X. Chen & Sullivan, 2003; Lorenz et al., 2001), sequence-based typing (Bochud et al., 2007; X. Chen & Sullivan, 2003), and allele-specific oligonucleotide hybridization (Rickert et al., 2005). The PCR-SSCP method developed could not only reveal the four sequences (*A* to *D*) that had been reported previously, but also revealed a further three new sequences (*E* to *G*) in a population of 1670 NZ sheep. Given this result, it is possible that PCR-SSCP approaches could also be readily adaptable to typing different *TLRs* in both sheep

and other species, and in the context of TLR4 typing it seems possible that further genetic variation might be found as more sheep of different breeds are typed.

The human and ovine TLR4 extracellular domain consists of 23 predicted LRRs that interact with different types of ligands (Ohara et al., 2006). The central region of the extracellular domain of human TLR4 (LRR9 to LRR14), is involved in binding putative co-receptors and ligands (Bell et al., 2003) and mutations in this region have been significantly associated with a response to LPS (Arbour et al., 2000) and with susceptibility to a variety of diseases (Hawn et al., 2005; Lorenz et al., 2002; Mockenhaupt et al., 2006). This suggests that variation in this region of *TLR4* in sheep may also affect the function of TLR4 in immune response.

The seven *TLR4* sequences (*A* to *G*) identified in the sheep studied, would if expressed, result in amino acid substitutions located in LRR13, 14 and 15 (Table 6.4), which contain a putative ligand binding region (amino acids 274-368). Most of the amino acid substitutions were conservative with the amino acid replaced by a similar type of amino acid. However, *TLR4 C* and *D* sequences would result in substitutions that were semi-conservative or radical (Lys344Asn, Asp363Gly and Asp395Tyr). Lys344Asn and Asp395Tyr are changes from charged to non-charged amino acids, while Asp363Gly is a change from a polar to a non-polar (hydrophobic) amino acid. The *TLR4 C* sequence contains all of these amino acid substitutions.

In humans, two *TLR4* missense mutations, Asp299Gly and Thr399Ile, have been associated with longevity (Balistreri et al., 2009). These mutations were found in LRR11 and 15, respectively (see table 6.2) and both of the mutations have been predicted to affect a ligand-binding and co-receptor-binding region, respectively (White et al., 2003). Although the effect of Asp299Gly and Thr399Ile mutations on TLR4 structure has not been well explained, Rallabhandi et al. (2006) have revealed a structural model of the extracelluar domain of TLR4 with these mutations. In this structure, the mutations lie on the same face of the scaffold and form a saddle-like structure that may provide a docking site for either ligand or co-receptor. Asp299Gly removes both a potential negative charge, and increases rotational freedom about the peptide bond, which may affect secondary structure immediately adjacent to this residue. Thus, this structural change in TLR4 could potentially affect ligand interaction.

Ferwerda et al. (2007) have suggested that in humans Asp299Gly and Thr399Ile mutations have been under evolutionary pressure from pathogens. They have found a high

variability in the occurrence of these mutations in different ethnic groups. Asp299Gly and Thr399Ile have been observed at a high frequency in African population, while population from Asia (Han Chinese, Indonesian, and Papuan) and America (Trio Indians in Surinam) had very low frequencies (Ferwerda et al., 2007). This suggests that the interaction between the host innate immune system and infectious disease pressures in particular environments may have shaped the genetic variation and function of our immune system during the "out-of-Africa" migration of modern humans.

In the sheep population studied, TLR4 Asp299Gly and Thr399Ile variation was not found. Position 299 was occupied by asparagine and 399 by threonine. This suggests these mutations are either very rare, or don't exist, and therefore they may not be an important factor in determining ovine longevity. However, in this study, the human missense mutation at amino acid 363 (Asp363Gly) was also observed in LRR14 of ovine *TLR4*. Asp363Gly could change the negative charge of LRR14, and this may affect PAMP recognition.

Ovine *TLR4* variation in exon 3 had no significant effect on longevity, but tends to be associated with fecundity in sheep. The presence of *TLR4 C* was associated with decreased fecundity when the model was corrected for "breed" (1.64 ± 0.03, $P = 0.004$), but not when corrected for flock. The presence of *TLR4 A* tended to decrease fecundity, when the model was corrected for "flock" (1.75 ± 0.02, $P = 0.066$). Breed and flock were confounded in this study because most flocks studied were of a single breed. If the association between *TLR4 C* and fecundity is to be believed, then it needs to be validated on a larger number of sheep, where breed and flock are not confounded. If the association is real, then potentially *TLR4* variation may be a useful marker of variation in fecundity in sheep.

Chapter 7
General summary and future directions

This thesis focused on assessing the genetic variation in potential longevity genes and its impact on longevity and fecundity traits in sheep. The genes studied were chosen based upon them having been shown to affect longevity or fecundity in humans and/or other species. The genes chosen were involved in either the control of energy metabolism (IGF1R and FOXO3; Chapter 3 and 4), protein degradation (CAST; Chapter 5) or immune response (TLR4; Chapter 6).

In studying sheep longevity it is necessary to investigate whether increasing their life-span is associated with a decrease in fertility or fecundity as would be suggested by "the trade-off" concept (Kirkwood & Holliday, 1979). In this study the relationship between longevity and fecundity in sheep was weak and negative (Chapter 2). This suggests that selection for increased longevity is unlikely have a major effect on ewe fecundity. Long-lived ewes could in effect provide both improved production and reproductive performance.

Three novel sequences (*A*, *B*, and *C*) of ovine *IGF1R* were identified in exon 3 of this gene and the presence of the *C* sequence was associated with increased ewe longevity (Chapter 3). This could be used as a potential way of improving sheep life-span, but only if further investigations confirm that the *C* sequence has no negative pleiotropic effect.

Ten nucleotide substitutions defining seven haplotypes (*A* to *G*) across the coding region of ovine *FOXO3* were identified and characterised. Haplotype *D* was subsequently associated with a decreased mean age in the sheep studied (Chapter 4). However, once again further study is required to ascertain how genetic variability in ovine *FOXO3* may specifically affect sheep longevity and whether the gene also either directly or indirectly impacts on factors that may lead to reduced productivity and/or an increased likelihood of culling.

An analysis of the extended haplotypes through the exon 6 to intron 12 region of ovine *CAST* revealed nine haplotypes, and subsequent analysis suggested that DNA recombination may have occurred (Chapter 5). It is still conceivable that even more genetic variation exists, as more sheep from different breeds are analysed and genotyping methods are developed that enable variation across the whole CAST gene to be easily assessed in large numbers of sheep. Thus, further study is needed to characterise the extent of *CAST* genetic diversity, and across a

large number of breeds. This would better facilitate more comprehensive study of associations between *CAST* variation and phenotypic variation.

No association was observed between variation in the ovine CAST gene and either longevity or fecundity in sheep studied (Chapter 5), but the *CAST* variation had a significant effect on birth weight (Chapter 5). This suggests that *CAST* might be a regulator of birth weight in sheep and this finding if better understood may enable better management of optimum birth weight and also potentially improve lamb survival.

A simple and reliable genotyping method for ovine *TLR4* variation was developed and readied for the study of large numbers of sheep (Chapter 6). Ovine *TLR4* variation sequence variants (*A* to *D*) in exon 3 had no significant effect on longevity, but tended to be associated with fecundity in sheep. The presence of *TLR4 A* and *TLR4 C* was associated with decreased fecundity when the model was corrected for "flock" (1.75 ± 0.02, $P = 0.066$) and "breed" (1.64 ± 0.03, $P = 0.004$), respectively. This result suggests that *TLR4* variation may be a useful marker of variation in fecundity in sheep. Further study with a larger number of samples in different breeds and flocks would be required to confirm this result.

Recent, advances in molecular genetics and statistical methodology have contributed to the identification and estimation of the effects of major genes in animal production, and their potential use in the genetic improvement of livestock. However, there is little research work the establishment of effective genetic selection methods for increased longevity. While the work presented in this thesis points to genetic variation in the IGF1R and FOXO3 genes as potential markers for longevity, and variation in CAST and TLR4 genes as potential markers for birth weight and fecundity respectively, these are certainly "early" findings and to have value they will first need to be reproduced and second the underlying biochemistry of the effect of the genetic variation will likely need greater understanding.

In the genetic association study, longevity data was collected from different breeds that belonged to 36 flocks of sheep. Few of the farmers had more than one breed and there is significant variation in longevity between flocks within a given breed, presumably reflecting different management policies for retaining or culling ewes on different farms. Given that most farms only have one breed of sheep, then farm and breed are conflicted. Correcting statistical models for both factors may have created a compounded error, thus either breed or flock, and not both, needs to be incorporated into in the GLMMs. Alternatively, future

research needs to be better orchestrated to remove the possibility of having confounded factors such as breed and farm effects.

It is clear that the results in this study need to be reproduced in order to minimise the potential error from breed and farm-related factors. This will be needed to illustrate that the associations haven't occurred by chance. It would be appropriate that further investigation involves a larger number of samples and with better control. While this is easy to say, the logistics of doing this may be challenging.

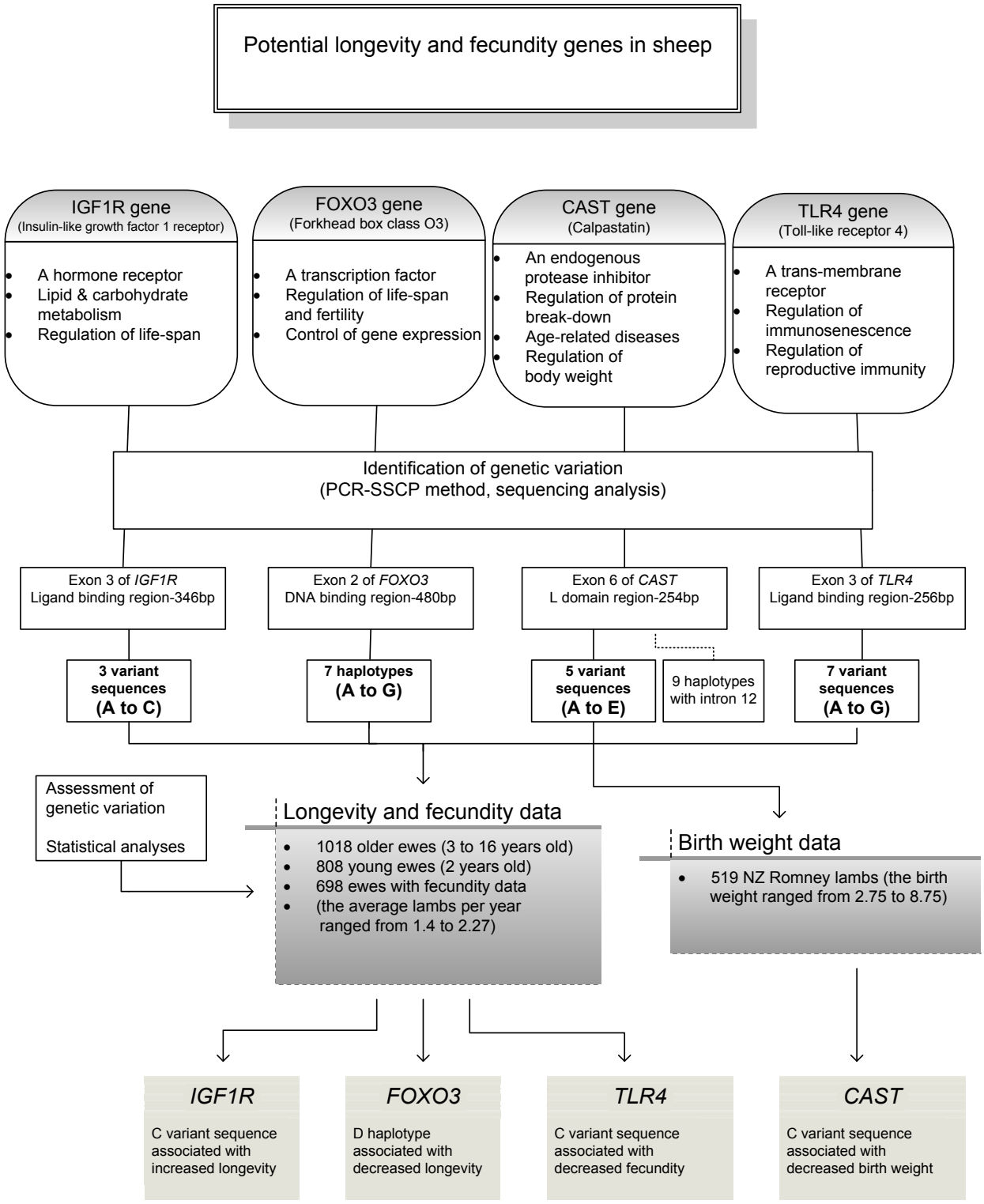
This study has investigated sheep longevity and its relationship with reproductive performance, because lamb is currently the main source of income for New Zealand farmers. It should be possible to obtain increase longevity and maintain the reproductive performance needed for economic profitability. However, future work needs to be undertaken to find if links exist between sheep longevity and other production traits.

In cattle, there is relationship between longevity and other production traits including the weight of the calf and its muscularity (Forabosco et al., 2004). These traits appear to be important in culling decisions and farmers seems to avoid animals that have calving difficulties and to retain muscle development. A similar system of culling decision-making occurs in both commercial and stud sheep farming; therefore it would also be worth investigating whether sheep longevity affects these traits.

Furthermore, teeth condition is also an important determinant of longevity in herbivorous animals, and it as reported healthy and long-lasting teeth are heritable (Mekkawy et al., 2009) in sheep, then this too needs to be considered in future design.

Finding genes involved in dental health would be valuable. For example, the transcription factor Ctip2 is a critical regulator of the enamel secreting cells during teeth development and growth (Golonzhka et al., 2009). Enamel is the outermost layer of the tooth and is important for the prevention of tooth decay or infection. Combined with the use of the potential longevity genes identified here, such a study could provide more accurate criteria for selecting sheep for longevity.

Diagrammatic summary of this thesis



References

Achyut, B. R., Ghoshal, U. C., Moorchung, N., & Mittal, B. (2007). Association of Toll-like receptor–4 (Asp299Gly and Thr399Ileu) gene polymorphisms with gastritis and precancerous lesions. *Human Immunology, 68*(11), 901-907.

Adams, T. E., Epa, V. C., Garrett, T. P. J., & Ward, C. W. (2000). Structure and function of the type 1 insulin-like growth factor receptor. *Cellular and Molecular Life Sciences, 57*(7), 1050-1093. doi:10.1007/pl00000744

Akisaka, M., Suzuki, M., & Inoko, H. (1997). Molecular genetic studies on DNA polymorphism of the HLA Class II genes associated with human longevity. *Tissue Antigens, 50*, 489-493.

Al-Regaiey, K. A., Masternak, M. M., Bonkowski, M., Sun, L., & Bartke, A. (2005). Long-lived growth hormone receptor knockout mice: interaction of reduced insulin-iike growth factor 1/insulin signaling and caloric restriction. *Endocrinology, 146*(2), 851-860. doi:10.1210/en.2004-1120

Alexander, G. (1984). Constraints to lamb survival. In 'reproduction in sheep'. *Academy of Sciences, Canberra, Australia*, 199-209.

Altomare, K., Greco, V., Bellizzi, D., Berardelli, M., Dato, S., DeRango, F., . . . De Benedictis, G. (2003). The allele (A) (-110) in the promoter region of the HSP70-1 gene is unfavorable to longevity in women. *Biogerontology, 4*(4), 215-220. doi:10.1023/a:1025182615693

Anderson, M. J., Viars, C. S., Czekay, S., Cavenee, W. K., & Arden, K. C. (1998). Cloning and characterization of three human forkhead genes that comprise an FKHR-like gene subfamily. *Genomics, 47*(2), 187-199.

Anderson, R. M., & Weindruch, R. (2007). Metabolic reprogramming in dietary restriction. *Interdisciplinary Topics in Gerontology, 35*, 18-38.

Andrzej, B. (2006). Long-lived Klotho mice: new insights into the roles of IGF-1 and insulin in aging. *Trends in Endocrinology & Metabolism, 17*(2), 33-35.

Anselmi, C. V., Malovini, A., Roncarati, R., Novelli, V., Villa, F., Condorelli, G., . . . Puca, A. A. (2009). Association of the FOXO3A locus with extreme longevity in a southern Italian centenarian study. *Rejuvenation Research, 12(2)*, 95-104.

Antebi, A. (2007). Genetics of aging in *Caenorhabditis elegans*. *PLoS Genetics, 3*, 1565-1571.

Arantes-Oliveira, N., Apfeld, J., Dillin, A., & Kenyon, C. (2002). Regulation of life-span by germ-line stem cells in *Caenorhabditis elegans*. *Science, 295*(5554), 502-505. doi:10.1126/science.1065768

Arbour, N. C., Lorenz, E., Schutte, B. C., Zabner, J., Kline, J. N., Jones, M., . . . Schwartz, D. A. (2000). TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. *Nature Genetics, 25*(2), 187-191.

Arden, K. C. (2008). FOXO animal models reveal a variety of diverse roles for FOXO transcription factors. *Oncogene, 27*(16), 2345-2350.

Arking, D. E., Krebsova, A., Macek, M., Arking, A., Mian, I. S., Fried, L., . . . Dietz, H. C. (2002). Association of human aging with a functional variant of klotho. *Proceedings of the National Academy of Sciences of the United States of America, 99*(2), 856-861. doi:10.1073/pnas.022484299

Asano, T., Fujishiro, M., Kushiyama, A., Nakatsu, Y., Yoneda, M., Kamata, H., & Sakoda, H. (2007). Role of phosphatidylinositol 3-kinase activation on insulin action and its alteration in diabetic conditions. *Biological & Pharmaceutical Bulletin, 30(9)*, 1610-1616.

Attaix, D., Combaret, L., Tilignac, T., & Taillandier, D. (1999). Adaptation of the ubiquitin-proteasome proteolytic pathway in cancer cachexia. *Molecular Biology Reports, 26*(1), 77-82. doi:10.1023/a:1006961919775

Atzmon, G., Rincon, M., Schechter, C. B., Shuldiner, A. R., Lipton, R. B., Bergman, A., & Barzilai, N. (2006). Lipoprotein genotype and conserved pathway for exceptional longevity in humans. *PLoS Biology, 4*(4), e113.

Averna, M., De Tullio, R., Salamino, F., Melloni, E., & Pontremoli, S. (1999). Phosphorylation of rat brain calpastatins by protein kinase C. *FEBS Letters, 450*(1–2), 13-16.

Averna, M., Stifanese, R., De Tullio, R., Passalacqua, M., Defranchi, E., Salamino, F., . . . Pontremoli, S. (2007). Regulation of calpain activity in rat brain with altered Ca2+ homeostasis. *Journal of Biological Chemistry, 282*(4), 2656-2665. doi:10.1074/jbc.M606919200

Baker, J., Liu, J. P., Robertson, E. J., & Efstratiadis, A. (1993). Role of insulin-like growth factors in embryonic and postnatal growth. *Cell, 75(1)*, 73-82.

Bakkenist, C. J., & Kastan, M. B. (2003). DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature, 421*(6922), 499-506. doi:http://www.nature.com/nature/journal/v421/n6922/suppinfo/nature01368_S1.html

Balcombe, N. R., & Sinclair, A. (2001). Ageing: definitions, mechanisms and the magnitude of the problem. *Best Practice & Research Clinical Gastroenterology, 15*(6), 835-849.

Balistreri, C., Colonna-Romano, G., Lio, D., Candore, G., & Caruso, C. (2009). TLR4 polymorphisms and ageing: implications for the pathophysiology of age-related diseases. *Journal of Clinical Immunology, 29*(4), 406-415. doi:10.1007/s10875-009-9297-5

Barbieri, M., Bonafè, M., Franceschi, C., & Paolisso, G. (2003). Insulin/IGF-I-signaling pathway: an evolutionarily conserved mechanism of longevity from yeast to humans. *American Journal of Physiology - Endocrinology And Metabolism, 285*(5), E1064-E1071. doi:10.1152/ajpendo.00296.2003

Barendse, W. (2002). DNA markers for meat tenderness. *International patent application No. PCT/AU02/00122 [international patent publication WO 02/064820 A1]*.

Barendse, W., Harrison, B. E., Hawken, R. J., Ferguson, D. M., Thompson, J. M., Thomas, M. B., & Bunch, R. J. (2007). Epistasis between calpain 1 and its Inhibitor calpastatin within breeds of cattle. *Genetics, 176*(4), 2601-2610. doi:10.1534/genetics.107.074328

Bartke, A., Bonkowski, M., & Masternak, M. (2008). How diet interacts with lonevity genes. *Hormones (Athens), 7(1)*, 17-23.

Bartke, A., & Brown-Borg, H. (2004). Life extension in the dwarf mouse. *Current Topics in Developmental Biology*, 189-225.

Barton-Davis, E. R., Shoturma, D. I., Musaro, A., Rosenthal, N., & Sweeney, H. L. (1998). Viral mediated expression of insulin-like growth factor I blocks the aging-related loss of skeletal muscle function. *Proceedings of the National Academy of Sciences of the United States of America, 95*(26), 15603-15607. doi:10.1073/pnas.95.26.15603

Barzilai, N., Atzmon, G., Schechter, C., Schaefer, E. J., Cupples, A. L., Lipton, R., . . . Shuldiner, A. R. (2003). Unique lipoprotein phenotype and genotype associated with exceptional longevity. *JAMA: The Journal of the American Medical Association, 290*(15), 2030-2040. doi:10.1001/jama.290.15.2030

Baudry, M., Simonson, L., Dubrin, R., & Lynch, G. (1986). A comparative study of soluble calcium-dependent proteolytic activity in brain. *Journal of Neurobiology, 17(1)*, 15-28.

Bauer, S., Müller, T., & Hamm, S. (2009). Pattern recognition by toll-like receptors. *Advances in Experimental Medicine and Biology, 653*, 15-34.

Beckman, K. B., & Ames, B. N. (1998). The free radical theory of aging matures. *Physiological Reviews, 78*(2), 547-581.

Beg, A. A. (2002). Endogenous ligands of toll-like receptors: implications for regulating inflammatory and immune responses. *Trends in Immunology, 23*(11), 509-512.

Bell, J. K., Mullen, G. E. D., Leifer, C. A., Mazzoni, A., Davies, D. R., & Segal, D. M. (2003). Leucine-rich repeats and pathogen recognition in toll-like receptors. *Trends in Immunology, 24*(10), 528-533.

Ben-Aharon, I., Ben-Yosef, D., Amit, A., & Shalgi, R. (2005). Expression and immunolocalization of the calpain-calpastatin system in the human oocyte. *Fertility and Sterility, 83*(6), 1807-1813.

Berneis, K., & Keller, U. (1996). Metabolic actions of growth hormone: direct and indirect. *Baillieres Clinical Endocrinology and Metabolism, 10(3)*, 337-352.

Berryman, D. E., Christiansen, J. S., Johannsson, G., Thorner, M. O., & Kopchick, J. J. (2008). Role of the GH/IGF-1 axis in life-span and health-span: Lessons from animal models. *Growth Hormone & IGF Research, 18*(6), 455-471.

Bertipaglia, I., & Carafoli, E. (2007). Calpains and human disease. *Sub-cellular Biochemistry, 45*, 29-53.

Beutler, B. (2000). Tlr4: central component of the sole mammalian LPS sensor. *Current Opinion in Immunology, 12*(1), 20-26.

Biggs, W. H., Meisenhelder, J., Hunter, T., Cavenee, W. K., & Arden, K. C. (1999). Protein kinase B/Akt-mediated phosphorylation promotes nuclear exclusion of the winged helix transcription factor FKHR1. *Proceedings of the National Academy of Sciences of the United States of America, 96*(13), 7421-7426. doi:10.1073/pnas.96.13.7421

Biswas, S., Harris, F., Dennison, S., Singh, J. P., & Phoenix, D. (2005). Calpains: enzymes of vision? . *Medical Science Monitor, 11(9)*(RA301-RA310).

Blair, H. T., & Garrick, D. J. (2007). Application of new technologies in sheep breeding. *New Zealand Journal of Agricultural Research, 50*(2), 89-102.

Blakesley, V. A., Scrimgeour, A., Esposito, D., & Le Roith, D. (1996). Signaling via the insulin-like growth factor-I receptor: Does it differ from insulin receptor signaling? *Cytokine & Growth Factor Reviews, 7*(2), 153-159.

Blander, J. M. (2008). Phagocytosis and antigen presentation: a partnership initiated by toll-like receptors. *Annals of the Rheumatic Diseases, 67*(Suppl 3), iii44-iii49. doi:10.1136/ard.2008.097964

Boccitto, M., & Kalb, R. G. (2011). Regulation of FOXO-dependnet transcription by post-translational modifications. *Current Drug Targets, 12(9)*, 1303-1310.

Bochud, P.-Y., Bochud, M., Telenti, A., & Calandra, T. (2007). Innate immunogenetics: a tool for exploring new frontiers of host defence. *The Lancet Infectious Diseases, 7*(8), 531-542.

Bonafè, M., Barbieri, M., Marchegiani, F., Olivieri, F., Ragno, E., Giampieri, C., . . . Paolisso, G. (2003). Polymorphic variants of insulin-like growth factor I (IGF-I) receptor and phosphoinositide 3-kinase genes affect IGF-I plasma levels and human longevity: Cues for an evolutionarily conserved mechanism of life-span control. *Journal of Clinical Endocrinology & Metabolism, 88*(7), 3299-3304. doi:10.1210/jc.2002-021810

Bonafè, M., Olivieri, F., Cavallone, L., Giovagnetti, S., Marchegiani, F., Cardelli, M., . . . Franceschi, C. (2001). A gender–dependent genetic predisposition to produce high levels of IL-6 is detrimental for longevity. *European Journal of Immunology, 31*(8), 2357-2361. doi:10.1002/1521-4141(200108)31:8<2357::aid-immu2357>3.0.co;2-x

Borgerhoj Mulder, M. (1988). Reproductives success in three Kipsigis cohorts. In *Reproductive success (ed. T. Clutton-Brock)* (pp. 419-435): Cambridge University press.

Boulton, T. G., Nye, S. H., Robbins, D. J., Ip, N. Y., Radzlejewska, E., Morgenbesser, S. D., . . . Yancopoulos, G. D. (1991). ERKs: A family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. *Cell, 65*(4), 663-675.

Bree, R., Stenson-Cox, C., Grealy, M., Byrnes, L., Gorman, A., & Samali, A. (2002). Cellular longevity: role of apoptosis and replicative senescence. *Biogerontology, 3*(4), 195-206. doi:10.1023/a:1016299812327

Brenkman, A. B., & Burgering, B. M. T. (2003). FoxO3a eggs on fertility and aging. *Trends in Molecular Medicine, 9*(11), 464-467.

Breusing, N., & Grune, T. (2008). Regulation of proteasome-mediated protein degradation during oxidative stress and aging. *Biological Chemistry, 389*(3), 203-209. doi:10.1515/bc.2008.029

Brien, F. D., Hebart, M. L., Smith, D. H., Edwards, J. E. H., Greeff, J. C., Hart, K. W., . . . van der Werf, J. H. J. (2010). Opportunities for genetic improvement of lamb survival. *Animal Production Science, 50*(12), 1017-1025. doi:http://dx.doi.org/10.1071/AN10121

Brown-Borg, H. M. (2007). Hormonal regulation of longevity in mammals. *Ageing Research Reviews, 6*(1), 28-45.

Brownawell, A. M., Kops, G. J. P. L., Macara, I. G., & Burgering, B. M. T. (2001). Inhibition of nuclear import by protein kinase B (Akt) regulates the subcellular distribution and activity of the forkhead transcription factor AFX. *Molecular and Cellular Biology, 21*(10), 3534-3546. doi:10.1128/mcb.21.10.3534-3546.2001

Browner, W. S., Kahn, A. J., Ziv, E., Reiner, A. P., Oshima, J., Cawthon, R. M., . . . Cummings, S. R. (2004). The genetics of human longevity. *The American Journal of Medicine, 117*(11), 851-860.

Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Juo, P., Hu, L. S., . . . Greenberg, M. E. (1999). Akt promotes cell survival by phosphorylating and inhibiting a forkhead transcription factor. *Cell, 96*(6), 857-868.

Brunet, A., Park, J., Tran, H., Hu, L. S., Hemmings, B. A., & Greenberg, M. E. (2001). Protein kinase SGK mediates survival signals by phosphorylating the forkhead transcription factor FKHRL1 (FOXO3a). *Molecular and Cellular Biology, 21*(3), 952-965. doi:10.1128/mcb.21.3.952-965.2001

Burgering, B. M. T. (2008). A brief introduction to FOXOlogy. *Oncogene, 27*(16), 2258-2262.

Butcher, S. K., & Lord, J. M. (2004). Stress responses and innate immunity: aging as a contributory factor. *Aging Cell, 3*(4), 151-160. doi:10.1111/j.1474-9728.2004.00103.x

Byun, S. O., Fang, Q., Zhou, H., & Hickford, J. G. H. (2009). An effective method for silver-staining DNA in large numbers of polyacrylamide gels. *Analytical Biochemistry, 385*(1), 174-175.

Calnan, D. R., & Brunet, A. (2008). The FoxO code. *Oncogene, 27*(16), 2276-2288.

Candore, G., Balistreri, C. R., Listì, F., Grimaldi, M. P., Vasto, S., Colonna-Romano, G., . . . Caruso, C. (2006a). Immunogenetics, gender, and longevity. *Annals of the New York Academy of Sciences, 1089*(1), 516-537. doi:10.1196/annals.1386.051

Candore, G., Colonna-Romano, G., Balistreri, C. R., Carlo, D. D., Grimaldi, M. P., Listì, F., . . . Caruso, C. (2006). Biology of longevity: role of the innate immune system. *Rejuvenation Research, 9*(1), 143-148. doi:10.1089/rej.2006.9.143

Canto, C., Gerhart-Hines, Z., Feige, J. N., Lagouge, M., Noriega, L., Milne, J. C., . . . Auwerx, J. (2009). AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity. *Nature, 458*(7241), 1056-1060. doi:http://www.nature.com/nature/journal/v458/n7241/suppinfo/nature07813_S1.html

Carlsson, P., & Mahlapuu, M. (2002). Forkhead transcription factors: key players in development and metabolism. *Developmental Biology, 250*(1), 1-23.

Carneiro, M., & Ferrand, N. (2007). Extensive intragenic recombination and patterns of linkage disequilibrium at the CSN3 locus in European rabbit. *Genetics Selection Evolution, 39*(3), 341 - 352.

Carrieri, G., Marzi, E., Olivieri, F., Marchegiani, F., Cavallone, L., Cardelli, M., . . . Franceschi, C. (2004). The G/C915 polymorphism of transforming growth factor β1 is associated with human longevity: a study in Italian centenarians. *Aging Cell, 3*(6), 443-448. doi:10.1111/j.1474-9728.2004.00129.x

Casas, E., White, S. N., Wheeler, T. L., Shackelford, S. D., Koohmaraie, M., Riley, D. G., . . . Smith, T. P. L. (2006). Effects of calpastatin and μ-calpain markers in beef cattle on tenderness traits. *Journal of Animal Science, 84*(3), 520-525.

Castrillon, D. H., Miao, L., Kollipara, R., Horner, J. W., & DePinho, R. A. (2003). Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a. *Science, 301(5630)*, 215-218.

Chapes, S. K., Mosier, D. A., Wright, A. D., & Hart, M. L. (2001). MHCII, Tlr4 and Nramp1 genes control host pulmonary resistance against the opportunistic bacterium *Pasteurella pneumotropica*. *Journal of Leukocyte Biology, 69*(3), 381-386.

Chen, L. C., Gordon, R. E., Laskin, J. D., & Laskin, D. L. (2007). Role of TLR4 in liver macrophage and endothelial cell responsiveness during acute endotoxemia. *Experimental and Molecular Pathology, 83*(3), 311-326.

Chen, X., & Sullivan, P. F. (2003). Single nucleotide polymorphism genotyping: biochemistry, protocol, cost and throughput. *Pharmacogenomics Journal, 3*(2), 77-96.

Cheng, I., Plummer, S. J., Casey, G., & Witte, J. S. (2007). Toll-like receptor 4 genetic variation and advanced prostate cancer risk. *Cancer Epidemiology Biomarkers & Prevention, 16*(2), 352-355. doi:10.1158/1055-9965.epi-06-0429

Choi, B. H., Lee, J. S., Jang, G. W., Lee, H. Y., Lee1, J. W., Lee, K. T., . . . Kim, T. H. (2006). Mapping of the porcine calpastatin gene and association study of its variance with economic traits in pigs. *Asian-Austrailan, Journal of Animal Science, 19(8)*, 1085-1089.

Christensen, K., Johnson, T. E., & Vaupel, J. W. (2006). The quest for genetic determinants of human longevity: challenges and insights. *Nature Reviews, Genetics, 7*(6), 436-448.

Chung, H. Y., Choi, B., Jang, G., Lee, K., Kim, H., Yoon, S., . . . Hines, H. (2007). Effect of variants in the ovine skeletal-muscle-specific calpain gene on body weight. *Journal of Applied Genetics, 48*(1), 63-68. doi:10.1007/bf03194658

Chung, H. Y., Davis, M. E., & Hines, H. C. (1999). A DNA polymorphism of the bovine calpastatin gene detected by SSCP analysis. *Animal Genetics, 30*(1), 80-81. doi:10.1046/j.1365-2052.1999.00323-20.x

Chung, H. Y., Davis, M. E., & Hines, H. C. (2001). Genetic variants detected by PCR–RFLP in intron 6 of the bovine calpastatin gene. *Animal Genetics, 32*(1), 53-53. doi:10.1046/j.1365-2052.2001.0647l.x

Ciobanu, D. C., Bastiaansen, J. W. M., Lonergan, S. M., Thomsen, H., Dekkers, J. C. M., Plastow, G. S., & Rothschild, M. F. (2004). New alleles in calpastatin gene are associated with meat quality traits in pigs. *Journal of Animal Science, 82*(10), 2829-2839.

Clancy, D. J., Gems, D., Harshman, L. G., Oldham, S., Stocker, H., Hafen, E., . . . Partridge, L. (2001). Extension of life-span by loss of CHICO, a *Drosophila* insulin receptor substrate protein. *Science, 292*(5514), 104-106. doi:10.1126/science.1057991

Clutton-Brock, T. H., Stevenson, I. R., Marrow, P., MacColl, A. D., Houston, A. I., & McNamara, J. M. (1996). Population fluctuations, reproductive costs and life-history tactics in female soay sheep. *Journal of Animal Ecology, 65*(6), 675-689.

Coelho, J., & Barbosa, P. F. (2006). In '8th World Congress on Genetics Applied to Livestock Production'. In *Belo Horizonte,Minas Gerais* (pp. 1-52)

Cong, M., Thompson, V. F., Goll, D. E., & Antin, P. B. (1998). The bovine calpastatin gene promoter and a new N-terminal region of the protein are targets for cAMP-dependent protein kinase activity. *Journal of Biological Chemistry, 273*(1), 660-666. doi:10.1074/jbc.273.1.660

Corva, P., Soria, L., Schor, A., Villarreal, E., Cenci, M. P., & Motter, M. (2007). Association of CAPN1 and CAST gene polymorphisms with meat tenderness in *Bos Taurus* beef cattle from Argentina. *Genetics and Molecular Biology, 30*, 1064-1069.

Cuervo, A. M., & Dice, J. F. (1998). How do intracellular proteolytic systems change with age? *Frontiers in Bioscience: a Journal and Virtual Library, 1*(3), d25-d43.

Cynthia, K. (2001). A conserved regulatory system for aging. *Cell, 105*(2), 165-168.

Datta, S. R., Brunet, A., & Greenberg, M. E. (1999). Cellular survival: a play in three Akts. *Genes & Development, 13*(22), 2905-2927.

David, L. L., Wright, J. W., & Shearer, T. R. (1992). Calpain II induced insolubilization of lens β-crystallin polypeptides may induce cataract. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease, 1139*(3), 210-216.

de Magalhães, J. P., & Faragher, R. G. A. (2008). Cell divisions and mammalian aging: integrative biology insights from genes that regulate longevity. *BioEssays, 30*(6), 567-578. doi:10.1002/bies.20760

De Martinis, M., Franceschi, C., Monti, D., & Ginaldi, L. (2005). Inflamm-ageing and life-long antigenic load as major determinants of ageing rate and longevity. *FEBS Letters, 579*(10), 2035-2039.

Dedieu, S., Mazères, G., Poussard, S., Brustis, J. J., & Cottin, P. (2003). Myoblast migration is prevented by a calpain-dependent accumulation of MARCKS. *Biology of the Cell, 95*(9), 615-623.

Dedieu, S., Poussard, S., Mazères, G., Grise, F., Dargelos, E., Cottin, P., & Brustis, J.-J. (2004). Myoblast migration is regulated by calpain through its involvement in cell attachment and cytoskeletal organization. *Experimental Cell Research, 292*(1), 187-200.

Demetrius, L. (2004). Caloric restriction, metabolic rate, and entropy. *The Journal of Gerontology Serious A, Biological Science and Medical Science, 59*, B902-915.

Denley, A., Wang, C. C., McNeil, K. A., Walenkamp, M. J. E., van Duyvenvoorde, H., Wit, J. M., . . . Forbes, B. E. (2005). Structural and functional characteristics of the Val44Met insulin-like growth factor I missense mutation: correlation with effects on growth and development. *Molecular Endocrinology, 19*(3), 711-721. doi:10.1210/me.2004-0409

Dijkers, P. F., Medema, R. H., Pals, C., Banerji, L., Thomas, N. S. B., Lam, E. W. F., . . . Coffer, P. J. (2000). Forkhead transcription factor FKHR-L1 modulates cytokine-dependent transcriptional regulation of p27KIP1. *Molecular and Cellular Biology, 20*(24), 9138-9148. doi:10.1128/mcb.20.24.9138-9148.2000

Dobson, H., Smith, R. F., Royal, M. D., Knight, C. H., & Sheldon, I. M. (2007). The high-producing dairy cow and its reproductive performance. *Reproduction in Domestic Animals, 42*, 17-23. doi:10.1111/j.1439-0531.2007.00906.x

Du, M., Zhu, M. J., Means, W. J., Hess, B. W., & Ford, S. P. (2004). Effect of nutrient restriction on calpain and calpastatin content of skeletal muscle from cows and fetuses. *Journal of Animal Science, 82*(9), 2541-2547.

Ducrocq, V. P., Quaas, R. L., Pollak, E. J., & Casella, G. (1988). Length of productive life of dairy cows.1.Justification of a Weibull model. *Journal of Dairy Science, 71*, 3061-3070.

Ducrocq, V. P., & Sölkner, J. (1998). Implementation of routine breeding value evaluation for longevity of dairy cows using survival analysis technique. *In Proc. 6th World Congress of Genetics Applied to Livestock Production., Armidale, Australia*, 359-362.

Dupont, J., & Holzenberger, M. (2003). Biology of insulin-like growth factors in development. *Birth Defects Research Part C: Embryo Today: Reviews, 69*(4), 257-271. doi:10.1002/bdrc.10022

Eckmann, L. (2006). Sensor molecules in intestinal innate immunity against bacterial infections. *Current Opinion in Gastroenterology, 22(2)*, 95-101.

Essl, A. (1998). Longevity in dairy cattle breeding: a review. *Livestock Production Science, 57*, 79-89.

Everett-Hincks, J. M., & Dodds, K. G. (2008). Management of maternal-offspring behavior to improve lamb survival in easy care sheep systems. *Journal of Animal Science, 86*(14 suppl), E259-E270. doi:10.2527/jas.2007-0503

Everett-Hincks, J. M., Dodds, K. G., & Kerslake, J. I. (2007). Parturition duration and birthing difficulty in twin and triplet lambs. *Proceedings of the New Zealand Society of Animal Production 67*, 55-60.

Everett-Hincks, J. M., Lopez-Villalobos, N., Blair, H. T., & Stafford, K. J. (2005b). The effect of maternal behavior score on lamb and litter survival. *Livestock Production Science, 93*, 51-61.

Everitt, A. V., & Le Couteur, D. G. (2007). Life extension by calorie restriction in humans. *Annals of the New York Academy of Sciences, 1114*(1), 428-433. doi:10.1196/annals.1396.005

Fazeli, A., Bruce, C., & Anumba, D. O. (2005). Characterization of toll-like receptors in the female reproductive tract in humans. *Human Reproduction, 20*(5), 1372-1378. doi:10.1093/humrep/deh775

Ferguson, M., Rebrin, I., Forster, M. J., & Sohal, R. S. (2008). Comparison of metabolic rate and oxidative stress between two different strains of mice with varying response to caloric restriction. *Experimental Gerontology, 43*(8), 757-763.

Ferwerda, B., McCall, M. B. B., Alonso, S., Giamarellos-Bourboulis, E. J., Mouktaroudi, M., Izagirre, N., . . . Netea, M. G. (2007). TLR4 polymorphisms, infectious diseases, and evolutionary pressure during migration of modern humans. *Proceedings of the National Academy of Sciences of the United States of America, 104*(42), 16645-16650. doi:10.1073/pnas.0704828104

Festa–Bianchet, M., & King, W. J. (2007). Age–related reproductive effort in bighorn sheep ewes. *Ecoscience, 14*(3), 318-322. doi:10.2980/1195-6860(2007)14[318:areibs]2.0.co;2

Fetterman, C., Rannala, B., & Walter, M. (2008). Identification and analysis of evolutionary selection pressures acting at the molecular level in five forkhead subfamilies. *BMC Evolutionary Biology, 8*(1), 261.

Finch, C. E., & Ruvkun, G. (2001). The genetics of aging. *Annual Review of Genomics and Human Genetics, 2*(1), 435-462. doi:10.1146/annurev.genom.2.1.435

Firth, S. M., & Baxter, R. C. (2002). Cellular actions of the insulin-like growth factor binding proteins. *Endocrine Reviews, 23*(6), 824-854. doi:10.1210/er.2001-0033

Flachsbart, F., Caliebe, A., Kleindorp, R., Blanché, H., von Eller-Eberstein, H., Nikolaus, S., . . . Nebel, A. (2009). Association of *FOXO3A* variation with human longevity confirmed in German centenarians. *Proceedings of the National Academy of Sciences of the United States of America, 106*(8), 2700-2705. doi:10.1073/pnas.0809594106

Flatt, T., Min, K.-J., D'Alterio, C., Villa-Cuesta, E., Cumbers, J., Lehmann, R., . . . Tatar, M. (2008). *Drosophila* germ-line modulation of insulin signaling and life-span. *Proceedings of the National Academy of Sciences of the United States of America, 105*(17), 6368-6373. doi:10.1073/pnas.0709128105

Fogarty, N. M. (1992). Breeding for reproductive performance. In 'Reproduction in sheep'. *Canberra Australian Academy of Science in conjuntion with the Australian Wool Corporation*, 226-233.

Forabosco, F., Groen, A. F., Bozzi, R., Van Arendonk, J. A. M., Filippini, F., Boettcher, P., & Bijma, P. (2004). Phenotypic relationships between longevity, type traits, and production in Chianina beef cattle. *Journal of Animal Science, 82*(6), 1572-1580.

Franceschi, C., BonafÈ, M., Valensin, S., Olivieri, F., De Luca, M., Ottaviani, E., & De Benedictis, G. (2000). Inflamm-aging: an evolutionary perspective on immunosenescence. *Annals of the New York Academy of Sciences 908*(1), 244-254. doi:10.1111/j.1749-6632.2000.tb06651.x

Friedman, D. B., & Johnson, T. E. (1988). Three mutants that extend both mean and maximum life-span of the Nematode, *Caenorhabditis elegans*, define the age-1 gene. *Journal of Gerontology, 43*(4), B102-B109. doi:10.1093/geronj/43.4.B102

Fukata, M., Michelsen, K. S., Eri, R., Thomas, L. S., Hu, B., Lukasek, K., . . . Abreu, M. T. (2005). Toll-like receptor 4 is required for intestinal response to epithelial injury and limiting bacterial translocation in a murine model of acute colitis. *American Journal of Physiology - Gastrointestinal and Liver Physiology, 288*(5), G1055-G1065. doi:10.1152/ajpgi.00328.2004

Fulop, T., Larbi, A., Douziech, N., Fortin, C., Guérard, K.-P., Lesur, O., . . . Dupuis, G. (2004). Signal transduction and functional changes in neutrophils with aging. *Aging Cell, 3*(4), 217-226. doi:10.1111/j.1474-9728.2004.00110.x

Furuyama, T., Nakazawa, T., Nakano, I., & Mori, N. (2000). Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues. *The Biochemical Journal, 349(Pt2)*, 629-634.

Gaillard, J.-M., Allaine, D., Pontier, D., Yoccoz, N. G., & Promislow, D. E. L. (1994). Senescence in natural populations of mammals: a reanalysis. *Evolution, 48*(2), 509-516.

Gajiwala, K. S., & Burley, S. K. (2000). Winged helix proteins. *Current Opinion in Structural Biology, 10*(1), 110-116.

Gallardo, T. D., John, G. B., Bradshaw, K., Welt, C., Reijo-Pera, R., Vogt, P. H., . . . Castrillon, D. H. (2008). Sequence variation at the human FOXO3 locus: a study of premature ovarian failure and primary amenorrhea. *Human Reproduction, 23*(1), 216-221. doi:10.1093/humrep/dem255

Garasto, S., Rose, G., Derango, F., Berardelli, M., Corsonello, A., Feraco, E., . . . De Benedictis, G. (2003). The study of APOA1, APOC3 and APOA4 variability in healthy ageing people reveals another paradox in the oldest old subjects. *Annals of Human Genetics, 67*(1), 54-62. doi:10.1046/j.1469-1809.2003.00008.x

Garcia, M. D., Michal, J. J., Gaskins, C. T., Reeves, J. J., Ott, T. L., Liu, Y., & Jiang, Z. (2006). Significant association of the calpastatin gene with fertility and longevity in dairy cattle. *Animal Genetics, 37*(3), 304-305. doi:10.1111/j.1365-2052.2006.01443.x

Gasco, V., Beccuti, G., Marotta, F., Benso, A., Granata, R., Broglio, F., & Ghigo, E. (2010). Endocrine and metabolic actions of ghrelin. *Endocrine Development, 17*(86-95).

Gerisch, B., Weitzel, C., Kober-Eisermann, C., Rottiers, V., & Antebi, A. (2001). A hormonal signaling pathway influencing *C. elegans* metabolism, reproductive development, and life-span. *Developmental Cell, 1*(6), 841-851.

Giannakou, M. E., & Partridge, L. (2007). Role of insulin-like signalling in *Drosophila* life-span. *Trends in Biochemical Sciences, 32*(4), 180-188.

Ginaldi, L., De Martinis, M., Monti, D., & Franceschi, C. (2005). Chronic antigenic load and apoptosis in immunosenescence. *Trends in Immunology, 26*(2), 79-84.

Giordano, M., Marchetti, C., Chiorboli, E., Bona, G., & Richiardi, P. M. (1997). Evidence for gene conversion in the generation of extensive polymorphism in the promoter of the growth hormone gene. *Human Genetics, 100*(2), 249-255. doi:10.1007/s004390050500

Girling, J. E., & Hedger, M. P. (2007). Toll-like receptors in the gonads and reproductive tract: emerging roles in reproductive physiology and pathology. *Immunology and Cell Biology, 85*(6), 481-489.

Glabe, C. G. (2008). Structural classification of toxic amyloid oligomers. *Journal of Biological Chemistry, 283*(44), 29639-29643. doi:10.1074/jbc.R800016200

Glotov, O. S., & Baranov, V. S. (2007). Genetic polymorphism and aging. *Advances in Gerontology, 20*, 35-55.

Golden, T. R., & Melove, S. (2007). Gene expression changes associated with aging in *C. elegans*. *WormBook: the online review of C. elegans biology, 12*, 1-12.

Goll, D. E., Shannon, J. D., Edmunds, T., Sathe, S. K., Kleese, W. C., & Nagainis, P. A. (1983). Properties and regulation of the calcium dependent proteinase. *Calcium-Binding Proteins*, 19-35.

Goll, D. E., Thompson, V. F., Li, H., Wei, W. E. I., & Cong, J. (2003). The calpain system. *Physiological Reviews, 83*(3), 731-801. doi:10.1152/physrev.00029.2002

Goll, D. E., Thompson, V. F., Taylor, R. G., & Christiansen, J. S. (1992). Role of the calpain system in muscle growth. *Biochimie, 74(3)*, 225-237.

Golonzhka, O., Metzger, D., Bornert, J.-M., Bay, B. K., Gross, M. K., Kioussi, C., & Leid, M. (2009). Ctip2/Bcl11b controls ameloblast formation during mammalian odontogenesis. *Proceedings of the National Academy of Sciences of the United States of America, 106*(11), 4278-4283. doi:10.1073/pnas.0900568106

Gomez-Mejiba, S. E., Zhai, Z., Akram, H., Pye, Q. N., Hensley, K., Kurien, B. T., . . . Ramirez, D. C. (2009). Inhalation of environmental stressors & chronic inflammation: Autoimmunity and neurodegeneration. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 674*(1-2), 62-72.

Gomez, C. R., Boehmer, E. D., & Kovacs, E. J. (2005). The aging innate immune system. *Current Opinion in Immunology, 17*(5), 457-462.

Green, W. C. H. (1990). Reproductive effort and associated costs in bison (Bison bison): do older mothers try harder? *Behavioral Ecology, 1*(2), 148-160. doi:10.1093/beheco/1.2.148

Greer, E. L., & Brunet, A. (2005). FOXO transcription factors at the interface between longevity and tumor suppression. *Oncogene, 24*(50), 7410-7425.

Greer, E. L., Oskoui, P. R., Banko, M. R., Maniar, J. M., Gygi, M. P., Gygi, S. P., & Brunet, A. (2007). The energy sensor AMP-activated protein kinase directly regulates the mammalian FOXO3 transcription factor. *Journal of Biological Chemistry, 282*(41), 30107-30119. doi:10.1074/jbc.M705325200

Grivennikov, S. I., Greten, F. R., & Karin, M. (2010). Immunity, inflammation, and cancer. *Cell, 140*(6), 883-899.

Groenen, P. J. T. A., Merck, K. B., De Jong, W. W., & Bloemendal, H. (1994). Structure and modifications of the junior chaperone α-crystallin. *European Journal of Biochemistry, 225*(1), 1-19. doi:10.1111/j.1432-1033.1994.00001.x

Guarente, L., & Kenyon, C. (2000). Genetic pathways that regulate ageing in model organisms. *Nature, 408*(6809), 255-262.

Gudex, B. W., Hickford, J. G. H., & Frampton, C. M. (2005). Sire-line differences in lamb losses from starvation-exposure. *Proceedings of the New Zealand Society of Animal Production, 65*, 186-190.

Guertin, D. A., & Sabatini, D. M. (2005). An expanding role for mTOR in cancer. *Trends in Molecular Medicine, 11*(8), 353-361.

Guevara-Aguirre, J., Rosenbloom, A. L., Vasconez, O., Martinez, V., Gargosky, S. E., Allen, L., & Rosenfeld, R. G. (1997). Two-year treatment of growth hormone (GH) receptor deficiency with recombinant insulin-like growth factor I in 22 children: comparison of two dosage levels and to GH-treated GH deficiency. *Journal of Clinical Endocrinology & Metabolism, 82*(2), 629-633. doi:10.1210/jc.82.2.629

Haim, K., Ben-Aharon, I., & Shalgi, R. (2006). Expression and immunolocalization of the calpain–calpastatin system during parthenogenetic activation and fertilization in the rat egg. *Reproduction, 131*(1), 35-43. doi:10.1530/rep.1.00697

Halbhuber, K. J., Zimmermann, N., Oehring, H., Stibenz, D., & Linss, W. (1987). Red blood cell aging-membrane skeleton alteration and IgG receptor expression. *Folia Histochemica et Cytobiologica, Polish Academy of Sciences, Polish Histochemical and Cytochemical Society, 25(2)*, 137-142.

Haldane, J. B. S. (1941). New paths in genetics. *London: Allen & Unwin*.

Hamel, S., Côté, S. D., Gaillard, J.-M., & Festa-Bianchet, M. (2009). Individual variation in reproductive costs of reproduction: high-quality females always do better. *Journal of Animal Ecology, 78*(1), 143-151. doi:10.1111/j.1365-2656.2008.01459.x

Hansen, M., Taubert, S., Crawford, D., Libina, N., Lee, S. J., & Kenyon, C. (2007). Life-span extension by conditions that inhibit translation in *Caenorhabditis elegans*. *Aging Cell, 6*(1), 95-110.

Harman, S. M. (2004). What do hormones have to do with aging? what does aging have to do with hormones? *Annals of the New York Academy of Sciences, 1019*(1), 299-308. doi:10.1196/annals.1297.051

Harris, B. L. (2005). Breeding dairy cows for the future in New Zealand. *New Zealand Veterinary Journal, 53*(6), 384-389. doi:10.1080/00480169.2005.36582

Harris, B. L., & Kolver, E. S. (2001). Review of holsteinization on intensive pastoral dairy farming in New Zealand. *Journal of Dairy Science, 84, Supplement*(0), E56-E61.

Harwood, S. M., Yaqoob, M. M., & Allen, D. A. (2005). Caspase and calpain function in cell death: bridging the gap between apoptosis and necrosis. *Annals of Clinical Biochemistry, 42*(6), 415-431. doi:10.1258/000456305774538238

Hawn, T. R., Verbon, A., Janer, M., Zhao, L. P., Beutler, B., & Aderem, A. (2005). Toll-like receptor 4 polymorphisms are associated with resistance to Legionnaires' disease. *Proceedings of the National Academy of Sciences of the United States of America, 102*(7), 2487-2489. doi:10.1073/pnas.0409831102

Hayashi, K., Tomita, Y., Maeda, Y., Shinagawa, Y., Inoue, K., & Hashizume, T. (1985). The rate of degradation of myofibrillar proteins of skeletal muscle in broiler and layer chickens estimated by N tau-methylhistidine in excreta. *The British Journal of Nutrition, 54(1)*, 157-163.

Hayes, B., Hagesæther, N., Ådnøy, T., Pellerud, G., Berg, P. R., & Lien, S. (2006). Effects on production traits of haplotypes among casein genes in Norwegian goats and evidence for a site of preferential recombination. *Genetics, 174*(1), 455-464. doi:10.1534/genetics.106.058966

Hazlett, L., & Wu, M. (2011). Defensins in innate immunity. *Cell and Tissue Research, 343*(1), 175-188. doi:10.1007/s00441-010-1022-4

Hebert, D. N., & Molinari, M. (2007). In and out of the ER: protein folding, quality control, degradation, and related human diseases. *Physiological Reviews, 87*(4), 1377-1408. doi:10.1152/physrev.00050.2006

Herath, S., Williams, E. J., Lilly, S. T., Gilbert, R. O., Dobson, H., Bryant, C. E., & Sheldon, I. M. (2007). Ovarian follicular cells have innate immune capabilities that modulate their endocrine function. *Reproduction, 134*(5), 683-693. doi:10.1530/rep-07-0229

Hessner, M. J., Dinauer, D. M., Kwiatkowski, R., Neri, B., & Raife, T. J. (2001). Age-dependent prevalence of vascular disease-associated polymorphisms among 2689 volunteer blood donors. *Clinical Chemistry, 47*(10), 1879-1884.

Higuchi, M., Iwata, N., & Saido, T. C. (2005). Understanding molecular mechanisms of proteolysis in Alzheimer's disease: Progress toward therapeutic interventions. *Biochimica et Biophysica Acta (BBA) - Proteins & Proteomics, 1751*(1), 60-67.

Hjelmborg, J., Iachine, I., Skytthe, A., Vaupel, J., McGue, M., Koskenvuo, M., . . . Christensen, K. (2006). Genetic influence on human life-span and longevity. *Human Genetics, 119*(3), 312-321. doi:10.1007/s00439-006-0144-y

Holliday, R. (2006). Aging is no longer an unsolved problem in biology. *Annals of the New York Academy of Sciences, 1067*(1), 1-9. doi:10.1196/annals.1354.002

Holzenberger, M., Dupont, J., Ducos, B., Leneuve, P., Geloen, A., Even, P. C., . . . Le Bouc, Y. (2003). IGF-1 receptor regulates life-span and resistance to oxidative stress in mice. *Nature, 421*(6919), 182-187. doi:http://www.nature.com/nature/journal/v421/n6919/suppinfo/nature01298_S1.html

Holzenberger, M., Hamard, G., Zaoui, R., Leneuve, P., Ducos, B., Beccavin, C., . . . Le Bouc, Y. (2001). Experimental IGF-I receptor deficiency generates a sexually dimorphic pattern of organ-specific growth deficits in mice, affecting fat tissue in particular. *Endocrinology, 142*(10), 4469-4478. doi:10.1210/en.142.10.4469

Honda, Y., & Honda, S. (1999). The daf-2 gene network for longevity regulates oxidative stress resistance and Mn-superoxide dismutase gene expression in *Caenorhabditis elegans*. *The FASEB Journal, 13*(11), 1385-1393.

Hoogland, J. L. (1995). The black-tailed prairie dog: social life of a burrowing mammal. *University of Chicago Press, Chicago, Illinois, USA*.

Horne, A. W., Stock, S. J., & King, A. E. (2008). Innate immunity and disorders of the female reproductive tract. *Reproduction, 135*(6), 739-749. doi:10.1530/rep-07-0564

Hornsby, P. J. (2011). Cellular aging and cancer. *Critical Reviews in Oncology/Hematology, 79*(2), 189-195.

Horwitz, J. (2003). Alpha-crystallin. *Experimental Eye Research, 76*(2), 145-153.

Hosaka, T., Biggs, W. H., Tieu, D., Boyer, A. D., Varki, N. M., Cavenee, W. K., & Arden, K. C. (2004). Disruption of forkhead transcription factor (FOXO) family members in mice reveals their functional diversification. *Proceedings of the National Academy of Sciences of the United States of America, 101*(9), 2975-2980. doi:10.1073/pnas.0400093101

Hsieh, P., & Yamane, K. (2008). DNA mismatch repair: Molecular mechanism, cancer, and ageing. *Mechanisms of Ageing and Development, 129*(7-8), 391-407.

Hsin, H., & Kenyon, C. (1999). Signals from the reproductive system regulate the life-span of *C. elegans*. *Nature, 399*(6734), 362-366.

Huang, Y., Henry, C. J., Dantzer, R., Johnson, R. W., & Godbout, J. P. (2008). Exaggerated sickness behavior and brain proinflammatory cytokine expression in aged mice in response to intracerebroventricular lipopolysaccharide. *Neurobiology of Aging, 29*(11), 1744-1753.

Hwangbo, D. S., Gersham, B., Tu, M.-P., Palmer, M., & Tatar, M. (2004). *Drosophila* dFOXO controls life-span and regulates insulin signalling in brain and fat body. *Nature, 429*(6991), 562-566. doi:http://www.nature.com/nature/journal/v429/n6991/suppinfo/nature02549_S1.html

Ibrahim, M., Upreti, R. K., & Kidwai, A. M. (1994). Calpain from rat intestinal epithelial cells: age-dependent dynamics during cell differentiation. *Molecular and Cellular Biochemistry, 131(1)*, 49-59.

Imran, S. A., Pelkey, M., Clarke, D. B., Clayton, D., Trainer, P., & Ezzat, S. (2010). Spuriously elevated serum IGF-1 in adult individuals with delayed puberty: a diagnostic pitfall. *International Journal of Endocrinology, 2010*. doi:10.1155/2010/370692

Jenniskens, Y. M., Koevoet, W., de Bart, A. C. W., Weinans, H., Jahr, H., Verhaar, J. A. N., . . . van Osch, G. J. V. M. (2006). Biochemical and functional modulation of the cartilage collagen network by IGF1, TGFβ2 and FGF2. *Osteoarthritis and Cartilage, 14*(11), 1136-1146.

Jiang, W. j. (2008). Sirtuins: Novel targets for metabolic disease in drug development. *Biochemical and Biophysical Research Communications, 373*(3), 341-344.

John, G. B., Shirley, L. J., Gallardo, T. D., & Castrillon, D. H. (2007). Specificity of the requirement for Foxo3 in primordial follicle activation. *Reproduction, 133*(5), 855-863. doi:10.1530/rep-06-0051

Johnson, T. M., Meade, K., Pathak, N., Marques, M. R., & Attardi, L. D. (2008). Knockin mice expressing a chimeric p53 protein reveal mechanistic differences in how p53 triggers apoptosis and senescence. *Proceedings of the National Academy of Sciences of the United States of America, 105*(4), 1215-1220. doi:10.1073/pnas.0706764105

Jones, K. G. (2004). Trends in the U.S. Sheep. *Electronic Report from the Economic Research Service, United States Department of Agriculture*.

Jorgenson, J. T., Festa-Bianchet, M., Gaillard, J.-M., & Wishart, W. D. (1997). Effects of age, sex disease, and density on survival of bighorn sheep. *Ecology, 78*(4), 1019-1032. doi:10.1890/0012-9658(1997)078[1019:eoasda]2.0.co;2

Jünger, M. A., Rintelen, F., Stocker, H., Wasserman, J. D., Végh, M., Radimerski, T., . . . Hafen, E. (2003). The *Drosophila* forkhead transcription factor FOXO mediates the reduction in cell number associated with reduced insulin signaling. *Journal of Biology, 2(3)*, 20.

Juszczuk-Kubiak, E., Wyszyńska-Koko, J., Wicińska, K., & Rosochacki, S. (2008). A novel polymorphisms in intron 12 of the bovine calpastatin gene. *Molecular Biology Reports, 35*(1), 29-35. doi:10.1007/s11033-006-9048-0

Kajava, A. V., Vassart, G., & Wodak, S. J. (1995). Modeling of the three-dimensional structure of proteins with the typical leucine-rich repeats. *Structure, 3(9)*, 867-877.

Karlsson, J. O., Blennow, K., Janson, I., Blomgren, K., Karlsson, I., Regland, B., . . . Gottfries, C. G. (1995). Increased proteolytic activity in lymphocytes from patients with early onset Alzheimer's disease. *Neurobiology of Aging, 16*(6), 901-906.

Kashii, Y., Uchida, M., Kirito, K., Tanaka, M., Nishijima, K., Toshima, M., . . . Komatsu, N. (2000). A member of Forkhead family transcription factor, FKHRL1, is one of the downstream molecules of phosphatidylinositol 3-kinase-Akt activation pathway in erythropoietin signal transduction. *Blood, 96*(3), 941-949.

Kawasaki, H., Emori, Y., Imajoh-Ohmi, S., Minami, Y., & Suzuki, K. (1989). Identification and characterization of inhibitory sequences in four repeating domains of the endogenous inhibitor for calcium-dependent protease. *Journal of Biochemistry, 106(2)*, 274-281.

Kay, M. M., Flowers, N., Goodman, J., & Bosman, G. (1989). Alteration in membrane protein band 3 associated with accelerated erythrocyte aging. *Proceedings of the National Academy of Sciences of the United States of America, 86*(15), 5834-5838.

Kelley, R. O., & Perdue, B. D. (1980). Development of the aging cell surface: Structural patterns of gap junction assembly between metabolic mutants and progressively subcultivated human diploid fibroblasts (IMR-90). *Experimental Gerontology, 15*(5), 407-421.

Kemp, C. M., Sensky, P. L., Bardsley, R. G., Buttery, P. J., & Parr, T. (2010). Tenderness – An enzymatic view. *Meat Science, 84*(2), 248-256.

Kennedy, B. K. (2008). The genetics of ageing: insight from genome-wide approaches in invertebrate model organisms. *Journal of Internal Medicine, 263*(2), 142-152. doi:10.1111/j.1365-2796.2007.01903.x

Kenyon, C. (2001). A conserved regulatory system for aging. *Cell, 105*(2), 165-168.

Kenyon, C. (2005). The plasticity of aging: insights from long-lived mutants. *Cell, 120*(4), 449-460.

Kenyon, C. (2011). The first long-lived mutants: discovery of the insulin/IGF-1 pathway for ageing. *Philosophical Transactions of the Royal Society B: Biological Sciences, 366*(1561), 9-16. doi:10.1098/rstb.2010.0276

Kenyon, C., Chang, J., Gensch, E., Rudner, A., & Tabtiang, R. (1993). A *C. elegans* mutant that lives twice as long as wild type. *Nature, 366*(6454), 461-464.

Kerslake, J. I., Everett-Hincks, J. M., & Campbell, A. W. (2005). Lamb survival: A new examination of an old problem. *Proceedings of the New Zealand Society of Animal Production, 65*(13-18).

Khorchid, A., & Ikura, M. (2002). How calpain is activated by calcium. *Nature Structural Biology, 9(4)*, 239-241.

Killefer, J., & Koohmaraie, M. (1994). Bovine skeletal muscle calpastatin: cloning, sequence analysis, and steady-state mRNA expression. *Journal of Animal Science, 72*(3), 606-614.

Kim, J. J., & Accili, D. (2002). Signalling through IGF-I and insulin receptors: where is the specificity? *Growth Hormone & IGF Research, 12*(2), 84-90.

Kirkwood, T. B. L. (1977). Evolution of ageing. *Nature, 270*, 301-304.

Kirkwood, T. B. L. (2002). Evolution of ageing. *Mechanisms of Ageing and Development, 123*(7), 737-745.

Kirkwood, T. B. L. (2005). Time of our lives. *EMBO Repprts, 6*(S1), S4-S8.

Kirkwood, T. B. L. (2011). Systems biology of ageing and longevity. *Philosophical Transactions of the Royal Society B: Biological Sciences, 366*(1561), 64-70. doi:10.1098/rstb.2010.0275

Kirkwood, T. B. L., & Austad, S. N. (2000). Why do we age? *Nature, 408*(6809), 233-238.

Kirkwood, T. B. L., & Holliday, R. (1979). The evolution of ageing and longevity. *Proceedings of the Royal Society of London, Series B: containing papers of a Biological Character, Royal Society (Great Britain), 205(1161)*, 531-546.

Klasing, K. C., Calvert, C. C., & Jarrell, V. L. (1987). Growth characteristics, protein synthesis, and protein degradation in muscles from fast and slow-growing chickens. *Poultry Science, 66(7)*, 1189-1196.

Kloet, D. E. A., & Burgering, B. M. T. (2011). The PKB/FOXO switch in aging and cancer. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 1813*(11), 1926-1937.

Knight, J. A. (2000). The biochemistry of aging. *Advances in Clinical Chemistry, 35*(1-62).

Kobe, B., & Deisenhofer, J. (1995). Proteins with leucine-rich repeats. *Current Opinion in Structural Biology, 5*(3), 409-416.

Koćwin-Podsiadła, M., Kurył, J., Krzęcio, E., Zybert, A., & Przybylsky, W. (2003). The interaction between calpastatin and RYR1gens for some pork quality traits. *Meat Science, 65*, 731-735.

Kojima, T., Kamei, H., Aizu, T., Arai, Y., Takayama, M., Nakazawa, S., . . . Hirose, N. (2004). Association analysis between longevity in the Japanese population and polymorphic variants of genes involved in insulin and insulin-like growth factor 1 signaling pathways. *Experimental Gerontology, 39*(11-12), 1595-1598.

Kops, G. J. P. L., Dansen, T. B., Polderman, P. E., Saarloos, I., Wirtz, K. W. A., Coffer, P. J., . . . Burgering, B. M. T. (2002). Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress. *Nature, 419*(6904), 316-321. doi:http://www.nature.com/nature/journal/v419/n6904/suppinfo/nature01036_S1.html

Krabbe, K. S., Pedersen, M., & Bruunsgaard, H. (2004). Inflammatory mediators in the elderly. *Experimental Gerontology, 39*(5), 687-699.

Krawinkel, U., Zoebelein, G., & Bothwell, A. L. (1986). Palindromic sequences are associated with sites of DNA breakage during gene conversion. *Nucleic Acids Research, 14(9)*, 3871-3882.

Kregel, K. C., & Zhang, H. J. (2007). An integrated view of oxidative stress in aging: basic mechanisms, functional effects, and pathological considerations. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology, 292*(1), R18-R36. doi:10.1152/ajpregu.00327.2006

Kumar, H., Kawai, T., & Akira, S. (2009). Pathogen recognition in the innate immune response. *Biochemical Journal, 420*(1), 1-16. doi:10.1042/bj20090272

Kuningas, M., de Rijk, R. H., Westendorp, R. G. J., Jolles, J., Eline Slagboom, P., & van Heemst, D. (2006). Mental performance in old age dependent on cortisol and genetic variance in the mineralocorticoid and glucocorticoid receptors. *Neuropsychopharmacology, 32*(6), 1295-1301.

Kurly, J., Kapelanski, W., & Pierzchala, M. (2003). Preliminary observations on the effect of calpastatin gene polymorphism in carcass trait in pig. *Animal Science Papers and Reports, 2*, 87-95.

Kyte, J., & Doolittle, R. F. (1982). A simple method for displaying the hydropathic character of a protein. *Journal of Molecular Biology, 157*(1), 105-132.

Larbi, A., Franceschi, C., Mazzatti, D., Solana, R., Wikby, A., & Pawelec, G. (2008). Aging of the immune system as a prognostic factor for human longevity. *Physiology, 23*(2), 64-74. doi:10.1152/physiol.00040.2007

Laron, Z. (2004). IGF1 and insulin as growth hormones. *Novartis Foundation Symposium, 262*(56-77; discussion 77-83, 256-258).

Larsen, P. L., Albert, P. S., & Riddle, D. L. (1995). Genes that regulate both development and longevity in *Caenorhabditis elegans*. *Genetics, 139*(4), 1567-1583.

Lehtinen, M. K., Yuan, Z., Boag, P. R., Yang, Y., Villén, J., Becker, E. B. E., . . . Bonni, A. (2006). A conserved MST-FOXO signaling pathway mediates oxidative-stress responses and extends life-span. *Cell, 125*(5), 987-1001.

Lenaz, G., D'Aurelio, M., Merlo Pich, M., Genova, M. L., Ventura, B., Bovina, C., . . . Parenti Castelli, G. (2000). Mitochondrial bioenergetics in aging. *Biochimica et Biophysica Acta (BBA) - Bioenergetics, 1459*(2-3), 397-404.

LeRoith, D., & Yakar, S. (2007). Mechanisms of Disease: metabolic effects of growth hormone and insulin-like growth factor 1. *Nature Clinical Practice Endocrinology & Metabolism, 3*(3), 302-310.

Leveque, G., Forgetta, V., Morroll, S., Smith, A. L., Bumstead, N., Barrow, P., . . . Malo, D. (2003). Allelic variation in TLR4 is linked to susceptibility to *Salmonella enterica Serovar typhimurium* Infection in Chickens. *Infection and Immunity, 71*(3), 1116-1124. doi:10.1128/iai.71.3.1116-1124.2003

Li, S., & Goldberg, E. (2000). A novel N-Terminal domain directs membrane localization of mouse testis-specific calpastatin. *Biology of Reproduction, 63*(6), 1594-1600. doi:10.1095/biolreprod63.6.1594

Li, Y., Wang, W.-J., Cao, H., Lu, J., Wu, C., Hu, F.-Y., . . . Tian, X.-L. (2009). Genetic association of FOXO1A and FOXO3A with longevity trait in Han Chinese populations. *Human Molecular Genetics, 18*(24), 4897-4904. doi:10.1093/hmg/ddp459

Li, Z., Cao, B., Zhao, B., Yang, X., Fan, M. Z., & Yang, J. (2009). Decreased expression of calpain and calpastatin mRNA during development is highly correlated with muscle protein accumulation in neonatal pigs. *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology, 152*(4), 498-503.

Liang, H., Masoro, E. J., Nelson, J. F., Strong, R., McMahan, C. A., & Richardson, A. (2003). Genetic mouse models of extended life-span. *Experimental Gerontology, 38*(11-12), 1353-1364.

Libby, P. (2007). Inflammatory mechanisms: the molecular basis of inflammation and disease. *Nutrition Reviews, 65*, S140-S146. doi:10.1111/j.1753-4887.2007.tb00352.x

Lin, K., Dorman, J. B., Rodan, A., & Kenyon, C. (1997). daf-16: An HNF-3/forkhead family member that can function to double the life-sapn of *Caenorhabditis elegans*. *Science, 278(5341)*, 1319-1322.

Linnebank, M., Fliessbach, K., Kolsch, H., Rietschel, M., & Wullner, U. (2005). The methionine synthase polymorphism c.2756A right curved arrow G (D919G) is

relevant for disease-free longevity. *International Journal of Molecular Medicine, 16(4)*, 759-761.

Lio, D., Balistreri, C. R., Colonna-Romano, G., Motta, M., Franceschi, C., Malaguarnera, M., . . . Caruso, C. (2002b). Association between the MHC class I gene HFE polymorphisms and longevity: a study in Sicilian population. *Genes and Immunity, 3*(1), 20-24.

Lio, D., Marino, V., Serauto, A., Gioia, V., Scola, L., Crivello, A., . . . Caruso, C. (2002a). Genotype frequencies of the +874T→A single nucleotide polymorphism in the first intron of the interferon-γ gene in a sample of Sicilian patients affected by tuberculosis. *European Journal of Immunogenetics, 29*(5), 371-374. doi:10.1046/j.1365-2370.2002.00327.x

Liton, P. B., Gonzalez, P., & Epstein, D. L. (2009). The role of proteolytic cellular systems in trabecular meshwork homeostasis. *Experimental Eye Research, 88*(4), 724-728.

Liu, J. L., Yakar, S., & Leroith, D. (2000). Conditional knockout of mouse insulin-like growth factor-1 gene using the Cre/loxP system. *Proceedings of the Society for Experimental Biology and Medicine, 223(4)*, 344-351.

Liu, J. P., Baker, J., Perkins, A. S., Robertson, E. J., & Efstratiadis, A. (1993). Mice carrying null mutations of the genes encoding insulin-like growth factor 1 (IGF1) and type 1 IGF receptor (IGF1R). *Cell, 75(1)*, 59-72.

Ljubuncic, P., & Reznick, A. A. (2009). The evolutionary theories of aging revisited-a mini-review. *Gerontology, 55*, 205-216.

Loison, A., Langvatn, R., & Solberg, E. J. (1999). Body size and mortality of red deer calves: disentangling sex and climate effects. *Ecography, 22*(22-30).

Lorenz, E., Frees, K. L., & Schwartz, D. A. (2001). Determination of the TLR4 genotype using allele-specific PCR. *Biotechniques, 31(1)*, 22-24.

Lorenz, E., Mira, J. P., Frees, K. L., & Schwartz, D. A. (2002). Relevance of mutations in the TLR4 receptor in patients with gram-negative septic shock. *Archives of Internal Medicine, 162*(9), 1028-1032. doi:10.1001/archinte.162.9.1028

Lu, Y.-C., Yeh, W.-C., & Ohashi, P. S. (2008). LPS/TLR4 signal transduction pathway. *Cytokine, 42*(2), 145-151.

Ludwig, T., Eggenschwiler, J., Fisher, P., D'Ercole, A. J., Davenport, M. L., & Efstratiadis, A. (1996). Mouse mutants lacking the type 2 IGF receptor (IGF2R) are rescued from perinatal lethality in Igf2and Igfr Null Backgrounds. *Developmental Biology, 177*(2), 517-535.

Lynch, G., Larson, J., & Baudry, M. (1986). In treatment development strategies for Alzheimer's disease. *Mark Powley Associates, Inc.Madison, CT*, 119-149.

Malcov, M., Ben-Yosef, D., Glaser, T., & Shalgi, R. (1997). Changes in calpain during meiosis in the rat egg. *Molecular Reproduction and Development, 48*(1), 119-126. doi:10.1002/(sici)1098-2795(199709)48:1<119::aid-mrd14>3.0.co;2-w

Martinelli, C. E., Custódio, R. J., & Aguiar-Oliveira, M. H. (2008). Fisiologia do eixo GH-sistema IGF. *Arquivos Brasileiros de Endocrinologia & Metabologia, 52*, 717-725.

Martinez-Vicente, M., Sovak, G., & Cuervo, A. M. (2005). Protein degradation and aging. *Experimental Gerontology, 40*(8-9), 622-633.

Masoro, E. J. (2005). Overview of caloric restriction and ageing. *Mechanisms of Ageing and Development, 126*(9), 913-922.

Matsushima, N., Tanaka, T., Enkhbayar, P., Mikami, T., Taga, M., Yamada, K., & Kuroki, Y. (2007). Comparative sequence analysis of leucine-rich repeats (LRRs) within vertebrate toll-like receptors. *BMC Genomics, 8*(1), 124.

Mauras, N., & Beaufrere, B. (1995). Recombinant human insulin-like growth factor-I enhances whole body protein anabolism and significantly diminishes the protein catabolic effects of prednisone in humans without a diabetogenic effect. *Journal of Clinical Endocrinology & Metabolism, 80*(3), 869-874. doi:10.1210/jc.80.3.869

Mauras, N., & Haymond, M. W. (1996). Metabolic effects of recombinant human-like growth factor-1: comparison with recombinant human growth hormone *Pediatrics Nephrology, 10(3)*, 318-323.

Mauras, N., & Haymond, M. W. (2005). Are the metabolic effects of GH and IGF-I separable? *Growth Hormone & IGF Research, 15*(1), 19-27.

McGregor, G. A. (2011). Incisor development, wear and loss in sheep and their impact on ewe production, longevity and economics: A review. *Small Ruminant Research, 95(2-3)*, 79-87.

Medema, R. H., Kops, G. J. P. L., Bos, J. L., & Burgering, B. M. T. (2000). AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. *Nature, 404*(6779), 782-787.

Medema, R. H., & Macurek, L. (2011). Checkpoint control and cancer. *Oncogene*.

Mekkawy, W., Roehe, R., Lewis, R. M., Davies, M. H., Bünger, L., Simm, G., & Haresign, W. (2009). Genetic relationship between longevity and objectively or subjectively assessed performance traits in sheep using linear censored models. *Journal of Animal Science, 87*(11), 3482-3489. doi:10.2527/jas.2008-1398

Mellgren, R. L., Lane, R. D., & Mericle, M. T. (1989). The binding of large calpastatin to biologic membranes is mediated in part by interaction of an amino terminal region with acidic phospholipids. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology, 999*(1), 71-77.

Melloni, E., Averna, M., Stifanese, R., De Tullio, R., Defranchi, E., Salamino, F., & Pontremoli, S. (2006). Association of Calpastatin with Inactive Calpain. *Journal of Biological Chemistry, 281*(34), 24945-24954. doi:10.1074/jbc.M601449200

Melloni, E., De Tullio, R., Averna, M., Tedesco, I., Salamino, F., Sparatore, B., & Pontremoli, S. (1998). Properties of calpastatin forms in rat brain. *FEBS Letters, 431*(1), 55-58.

Melloni, E., Salamino, F., & Sparatore, B. (1992). The calpain-calpastatin system in mammalian cells: properties and possible functions. *Biochemie, 74(3)*, 217-223.

Merry, B. J. (2005). Dietary restriction in rodents—delayed or retarded ageing? *Mechanisms of Ageing and Development, 126*(9), 951-959.

Miller, R. A. (2009). Cell stress and aging: new emphasis on multiplex resistance mechanisms. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences, 64A*(2), 179-182. doi:10.1093/gerona/gln072

Minobe, E., Hao, L.-Y., Saud, Z. A., Xu, J.-J., Kameyama, A., Maki, M., . . . Kameyama, M. (2006). A region of calpastatin domain L that reprimes cardiac L-type Ca2+ channels. *Biochemical and Biophysical Research Communications, 348*(1), 288-294.

Miyake, K. (2007). Innate immune sensing of pathogens and danger signals by cell surface Toll-like receptors. *Seminars in Immunology, 19*(1), 3-10.

Mockenhaupt, F. P., Cramer, J. P., Hamann, L., Stegemann, M. S., Eckert, J., Oh, N.-R., . . . Schumann, R. R. (2006). Toll-like receptor (TLR) polymorphisms in African children: Common TLR4 variants predispose to severe malaria. *Proceedings of the National Academy of Sciences of the United States of America, 103*(1), 177-182. doi:10.1073/pnas.0506803102

Morris, J. Z., Tissenbaum, H. A., & Ruvkun, G. (1996). A phosphatidylinositol-3-OH kinase family member regulating longevity and diapause in *Caenorhabditis elegans*. *Nature, 382*(6591), 536-539.

Mukhopadhyay, A., Oh, S. W., & Tissenbaum, H. A. (2006). Worming pathways to and from DAF-16/FOXO. *Experimental Gerontology, 41*(10), 928-934.

Murphy, C. T. (2006). The search for DAF-16/FOXO transcriptional targets: Approaches and discoveries. *Experimental Gerontology, 41*(10), 910-921.

Murphy, C. T., McCarroll, S. A., Bargmann, C. I., Fraser, A., Kamath, R. S., Ahringer, J., . . . Kenyon, C. (2003). Genes that act downstream of DAF-16 to influence the life-span of

Caenorhabditis elegans. *Nature, 424*(6946), 277-283. doi:http://www.nature.com/nature/journal/v424/n6946/suppinfo/nature01789_S1.html

Mushegian, A., & Medzhitov, R. (2001). Evolutionary perspective on innate immune recognition. *The Journal of Cell Biology, 155*(5), 705-710. doi:10.1083/jcb.200107040

Nakae, J., Cao, Y., Daitoku, H., Fukamizu, A., Ogawa, W., Yano, Y., & Hayashi, Y. (2006). The LXXLL motif of murine forkhead transcription factor FoxO1 mediates Sirt1-dependent transcriptional activity. *The Journal of Clinical Investigation, 116*(9), 2473-2483.

Nassiry, M. R., Tahmoures, P. M., Javad, M. A., Soltani, M., & Foroutani, F. S. (2006). Calpastatin polymorphism and its association with daily gain in kurdi sheep. *Journal of Biotechnology, 4(3)*, 188-192.

Netea, M. G., van de Veerdonk, F. L., Kullberg, B. J., Van der Meer, J. W. M., & Joosten, L. A. B. (2008). The role of NLRs and TLRs in the activation of the inflammasome. *Expert Opinion on Biological Therapy, 8*(12), 1867-1872. doi:doi:10.1517/14712590802494212

Newton, I., & Rothery, P. (1997). Senescence and reproductive value in sparrowhawks. *Ecology, 78*(4), 1000-1008. doi:10.1890/0012-9658(1997)078[1000:sarvis]2.0.co;2

Nixon, R. A. (1989). Calcium-activated neutral proteinases as regulators of cellular function implications for Alzheimer's disease pathogenesisa. *Annals of the New York Academy of Sciences, 568*(1), 198-208. doi:10.1111/j.1749-6632.1989.tb12509.x

Nixon, R. A., Saito, K. I., Grynspan, F., Griffin, W. R., Katayama, S., Honda, T., . . . Beermann, M. (1994). Calcium-activated neutral proteinase (calpain) system in aging and Alzheimer's disease. *Annals of the New York Academy of Sciences, 747*(1), 77-91. doi:10.1111/j.1749-6632.1994.tb44402.x

Nugent, R. A., & Jenkins, T. G. (1992). Effects of alternative lamb production systems, maternal line, and culling strategy on flock age structure. *Journal of Animal Science, 70*(8), 2285-2295.

Obsil, T., & Obsilova, V. (2000). Structure/function relationships underlying regulation of FOXO transcription factors. *Oncogene, 27*(16), 2263-2275.

Ogg, S., Paradis, S., Gottlieb, S., Patterson, G. I., Lee, L., Tissenbaum, H. A., & Ruvkun, G. (1997). The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in *C. elegans*. *Nature, 389*(6654), 994-999.

Ohara, T., Morishita, T., Suzuki, H., & Hibi, T. (2006). Heterozygous Thr135Ala polymorphism at leucine-rich repeat (LRR) in genomic DNA of toll-like receptor 4 in patients with poorly-differentiated gastric adenocarcinomas. *International Journal of Molecular Medicine, 18*, 59-63.

Ohlsson, C., Sjögren, K., Jansson, J. O., & Lsaksson, O. G. (2000). The relative importance of endocrine versus autocirne/paracrine insulin-like growth factor-1 in the regulation of body growth. *Pediatric Nephrology, 14(7)*, 541-543.

Oliveira, G., Tahara, E., Gombert, A., Barros, M., & Kowaltowski, A. (2008). Increased aerobic metabolism is essential for the beneficial effects of caloric restriction on yeast life-span. *Journal of Bioenergetics and Biomembranes, 40*(4), 381-388. doi:10.1007/s10863-008-9159-5

Ono, K., Condron, M. M., & Teplow, D. B. (2009). Structure–neurotoxicity relationships of amyloid β-protein oligomers. *Proceedings of the National Academy of Sciences of the United States of America, 106*(35), 14745-14750. doi:10.1073/pnas.0905127106

Orr, M. B., Christiansen, K. H., & Kissling, R. C. (1986). A survey of excessively worn incisors and periodontal disease in sheep *In Dunedin City, Silverpeak, Bruce and Clutha Counties.N.Z.Vet.J., 34*, 111-115.

Orwig, K. E., Bertrand, J. E., Ou, B. R., Forsberg, N. E., & Stormshak, F. (1994). Involvement of protein kinase-C, calpains, and calpastatin in prostaglandin F2 alpha-

induced oxytocin secretion from the bovine corpus luteum. *Endocrinology, 134*(1), 78-83. doi:10.1210/en.134.1.78

Ostan, R., Bucci, L., Capri, M., Salvioli, S., Scurti, M., Pini, E., . . . Franceschi, C. (2008). Immunosenescence and immunogenetics of human longevity. *Neuroimmunomodulation, 15*(224-240).

Ouban, A., Muraca, P., Yeatman, T., & Coppola, D. (2003). Expression and distribution of insulin-like growth factor-1 receptor in human carcinomas. *Human Pathology, 34*(8), 803-808.

Paaby, A. B., & Schmidt, P. S. (2009). Dissecting the genetics of longevity in *Drosophila melanogaster*. *Fly(Austin), 3*(1), 29-38.

Palladino, M. A., Johnson, T. A., Gupta, R., Chapman, J. L., & Ojha, P. (2007). Members of the toll-like receptor family of innate immunity pattern-recognition receptors are abundant in the male rat reproductive tract. *Biology of Reproduction, 76*(6), 958-964. doi:10.1095/biolreprod.106.059410

Pallás, M., Verdaguer, E., Tajes, M., Gutierrez-Cuesta, J., & Camins, A. (2008). Modulation of sirtuins: new targets for antiageing. *Recent Patents on CNS Drug Discovery, 3(1)*, 61-69.

Palmer, B. R., Roberts, N., Hickford, J. G., & Bickerstaffe, R. (1998). Rapid communication: PCR-RFLP for MspI and NcoI in the ovine calpastatin gene. *Journal of Animal Science, 76*(5), 1499-1500.

Palmer, B. R., Su, H. Y., Roberts, N., Hickford, J. G. H., & Bickerstaffe, R. (2000). Short communication: Single nucleotide polymorphisms in an intron of the ovine calpastatin gene. *Animal Biotechnology, 11*(1), 63-67. doi:10.1080/10495390009525948

Pamplona, R., & Barja, G. (2006). Mitochondrial oxidative stress, aging and caloric restriction: The protein and methionine connection. *Biochimica et Biophysica Acta (BBA) - Bioenergetics, 1757*(5-6), 496-508.

Parmley, J. L., Chamary, J. V., & Hurst, L. D. (2006). Evidence for purifying selection against synonymous mutations in mammalian exonic splicing enhancers. *Molecular Biology and Evolution, 23*(2), 301-309. doi:10.1093/molbev/msj035

Partridge, L., & Bruning, J. C. (2008). Forkhead transcription factors and ageing. *Oncogene, 27*(16), 2351-2363.

Partridge, L., & Gems, D. (2002). Mechanisms of aging: public or private? *Nature Reviews, Genetics, 3*(3), 165-175.

Partridge, L., Piper, M. D. W., & Mair, W. (2005). Dietary restriction in *Drosophila*. *Mechanisms of Ageing and Development, 126*(9), 938-950.

Pascal, C. (2011). Researches regarding quality of sheep skins obtained from Karakul from Botosani sheep. *Biotechnology in Animal Husbandry, 27*(3), 1123-1130.

Pasterkamp, G., Van Keulen, J. K., & De Kleijn, D. P. V. (2004). Role of Toll-like receptor 4 in the initiation and progression of atherosclerotic disease. *European Journal of Clinical Investigation, 34*(5), 328-334. doi:10.1111/j.1365-2362.2004.01338.x

Patel, Y. M., & Lane, M. D. (1999). Role of calpain in adipocyte differentiation. *Proceedings of the National Academy of Sciences of the United States of America, 96*(4), 1279-1284. doi:10.1073/pnas.96.4.1279

Pawelec, G. (2003). Immunosenescence and human longevity. *Biogerontology, 4*(3), 167-170. doi:10.1023/a:1024185405946

Pawlikowska, L., Hu, D., Huntsman, S., Sung, A., Chu, C., Chen, J., . . . for the Study of Osteoporotic, F. (2009). Association of common genetic variation in the insulin/IGF1 signaling pathway with human longevity. *Aging Cell, 8*(4), 460-472. doi:10.1111/j.1474-9726.2009.00493.x

Pearl, R. (1928). *The rate of living: being an account of some experimental studies on biology of life duration*. Knopf, New York

Pedersen, B. K., & Bruunsgaard, H. (2003). Possible beneficial role of exercise in modulating low-grade inflammation in the elderly. *Scandinavian Journal of Medicine & Science in Sports, 13*(1), 56-62. doi:10.1034/j.1600-0838.2003.20218.x

Perls, T. (2002). Genetic and environmental influences on exceptional longevity and the age nomogram. *Annals of the New York Academy of Sciences, 959*(1), 1-13. doi:10.1111/j.1749-6632.2002.tb02077.x

Phillips, L. S., Harp, J. B., Goldstein, S., Klein, J., & Pao, C. I. (1990). Regulation and action of insulin-like growth factors at the cellular level. *Proceedings of the Nutrition Society, 49*(03), 451-458. doi:doi:10.1079/PNS19900053

Piper, M. D. W., Selman, C., McElwee, J. J., & Partridge, L. (2008). Separating cause from effect: how does insulin/IGF signalling control life-span in worms, flies and mice? *Journal of Internal Medicine, 263*(2), 179-191. doi:10.1111/j.1365-2796.2007.01906.x

Platt, D. (1979). Theories and models of the biology of aging. *Aktuelle Gerontologie, 9*(163-166).

Pollack, M., Phaneuf, S., Dirks, A., & Leeuwenburgh, C. (2002). The role of apoptosis in the normal aging brain, skeletal muscle, and heart. *Annals of the New York Academy of Sciences, 959*(1), 93-107. doi:10.1111/j.1749-6632.2002.tb02086.x

Puca, A. A., Chatgilialoglu, C., & Ferreri, C. (2008). Lipid metabolism and diet: Possible mechanisms of slow aging. *The International Journal of Biochemistry & Cell Biology, 40*(3), 324-333.

Qu, H.-Q., Lawrence, S., Guo, F., Majewski, J., & Polychronakos, C. (2006). Strand bias in complementary single-nucleotide polymorphisms of transcribed human sequences: evidence for functional effects of synonymous polymorphisms. *BMC Genomics, 7*(1), 213.

Quarrie, J. K., & Riabowol, K. T. (2004). Murine models of life-span extension. *Science of Aging Knowledge Environment, 2004*(31), re5-. doi:10.1126/sageke.2004.31.re5

Rahmutula, D., Nakayama, T., Izumi, Y., Ozawa, Y., Shimabukuro, H., Kawamura, H., . . . Hen-Chen, Z. (2002). Angiotension-converting enzyme gene and longevity in the Xin Jiang Uighur autonomous region of China: an association study. *The Journal of Gerontology Serious A, Biological Science and Medical Science, 57(1)*, M57-60.

Rallabhandi, P., Bell, J., Boukhvalova, M. S., Medvedev, A., Lorenz, E., Arditi, M., . . . Vogel, S. N. (2006). Analysis of TLR4 polymorphic variants: new insights into TLR4/MD-2/CD14 stoichiometry, structure, and signaling. *The Journal of Immunology, 177*(1), 322-332.

Rao, M. V., Mohan, P. S., Peterhoff, C. M., Yang, D.-S., Schmidt, S. D., Stavrides, P. H., . . . Nixon, R. A. (2008). Marked calpastatin (CAST) depletion in Alzheimer's disease accelerates cytoskeleton disruption and neurodegeneration: neuroprotection by CAST overexpression. *The Journal of Neuroscience, 28*(47), 12241-12254. doi:10.1523/jneurosci.4119-08.2008

Rattan, S. I. S. (2004). Aging, anti-aging, and hormesis. *Mechanisms of Ageing and Development, 125*(4), 285-289.

Rattan, S. I. S. (2006). Theories of biological aging: Genes, proteins, and free radicals. *Free Radical Research, 40*(12), 1230-1238. doi:doi:10.1080/10715760600911303

Rattan, S. I. S. (2010). Synthesis, modification and turnover of proteins during aging. *Advances in Experimental Medicine and Biology, 694*, 1-13.

Ravulapalli, R., Campbell, R. L., Gauthier, S. Y., Dhe-Paganon, S., & Davies, P. L. (2009). Distinguishing between calpain heterodimerization and homodimerization. *FEBS Journal, 276*(4), 973-982. doi:10.1111/j.1742-4658.2008.06833.x

Reddy, G. B., Das, K. P., Petrash, J. M., & Surewicz, W. K. (2000). Temperature-dependent chaperone activity and structural properties of human αA- and αB-crystallins. *Journal of Biological Chemistry, 275*(7), 4565-4570. doi:10.1074/jbc.275.7.4565

Reiner, A. P., Diehr, P., Browner, W. S., Humphries, S. E., Jenny, N. S., Cushman, M., . . . Psaty, B. M. (2005). Common promoter polymorphisms of inflammation and thrombosis genes and longevity in older adults: The cardiovascular health study. *Atherosclerosis, 181*(1), 175-183.

Reinhardt, H. C., & Schumacher, B. (2012). The p53 network: cellular and systemic DNA damage responses in aging and cancer. *Trends in Genetics, 28*(3), 128-136.

Rena, G., Prescott, A. R., Guo, S., Cohen, P., & Unterman, T. G. (2001). Roles of the forkhead in rhabdomyosarcoma(FKHR) phosphorylation sites in regulating 14-3-3 binding, transactivation and nuclear targetting. *The Biochemical Journal, 354(Pt3)*, 605-612.

Richards, S., Watanabe, C., Santos, L., Craxton, A., & Clark, E. A. (2008). Regulation of B-cell entry into the cell cycle. *Immunological Reviews, 224*(1), 183-200. doi:10.1111/j.1600-065X.2008.00652.x

Richardson, A., Liu, F., Adamo, M. L., Remmen, H. V., & Nelson, J. F. (2004). The role of insulin and insulin-like growth factor-I in mammalian ageing. *Best Practice & Research: Clinical Endocrinology & Metabolism, 18*(3), 393-406.

Rickert, A. M., Ballvora, A., Matzner, U., Klemm, M., & Gebhardt, C. (2005). Quantitative genotyping of single-nucleotide polymorphisms by allele-specific oligonucleotide hybridization on DNA microarrays. *Biotechnology and Applied Biochemistry, 42(Pt1)*, 93-96.

Ricklefs, R. E. (1998). Evolutionary theories of aging: confirmation of a fundamental prediction, with implications for the genetic basis and evolution of life-span. *The American Naturalist, 152*(1), 24-44.

Ricklefs, R. E., & Cadena, C. D. (2008). Heritability of longevity in captive populations of nondomesticated mammals and birds. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences, 63*(5), 435-446.

Rink, L., Cakman, I., & Kirchner, H. (1998). Altered cytokine production in the elderly. *Mechanisms of Ageing and Development, 102(2-3)*, 199-209.

Rivett, A. J. (1990). Intracellular protein degradation. *Essays in Biochemistry, 25*, 39-81.

Robbins, D. J., Cheng, M., Zhen, E., Vanderbilt, C. A., Feig, L. A., & Cobb, M. H. (1992). Evidence for a Ras-dependent extracellular signal-regulated protein kinase (ERK) cascade. *Proceedings of the National Academy of Sciences of the United States of America, 89(15)*, 6924-6928.

Roberts, N., Palmer, B., Hickford, J. G., & Bickerstaffe, R. (1996). PCR-SSCP in the ovine calpastatin gene. *Animal Genetics, 27(3)*, 211.

Rodrigues, A., Queiróz, D. B. C., Honda, L., Silva, E. J. R., Hall, S. H., & Avellar, M. C. W. (2008). Activation of toll-like receptor 4 (TLR4) by in vivo and in vitro exposure of rat epididymis to lipopolysaccharide from *Escherichia Coli*. *Biology of Reproduction, 79*(6), 1135-1147. doi:10.1095/biolreprod.108.069930

Rogol, A. D., Roemmich, J. N., & Clark, P. A. (2002). Growth at puberty. *Journal of Adolescent Health, 31*(6, Supplement), 192-200.

Rojas, F. J., Brush, M., & Moretti-Rojas, I. (1999). Calpain–calpastatin: a novel, complete calcium-dependent protease system in human spermatozoa. *Molecular Human Reproduction, 5*(6), 520-526. doi:10.1093/molehr/5.6.520

Rosenberger, R. F. (1991). Senescence and the accumulation of abnormal proteins. *Mutation Research, 256(2-6)*, 255-262.

Russell, S. J., & Kahn, C. R. (2007). Endocrine regulation of ageing. *Nature Reviews Molecular Cell Biology, 8*(9), 681-691.

Salustri, A., Garlanda, C., Hirsch, E., De Acetis, M., Maccagno, A., Bottazzi, B., . . . Mantovani, A. (2004). PTX3 plays a key role in the organization of the cumulus oophorus extracellular matrix and in in vivo fertilization. *Development, 131*(7), 1577-1586. doi:10.1242/dev.01056

Sandri, M., & Carraro, U. (1999). Apoptosis of skeletal muscles during development and disease. *The International Journal of Biochemistry & Cell Biology, 31*(12), 1373-1390.

Sanguinetti, C. J., Dias Neto, E., & Simpson, A. J. (1994). Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. *Biotechniques, 17(5)*, 914-921.

Scales, G. H., Burton, R. N., & Moss, R. A. (1986). Lamb mortality, birthweight, and nutrition in late pregnancy. *New Zealand Journal of Agricultural Research, 29*, 75-82.

Schenkel, F. S., Miller, S. P., Jiang, Z., Mandell, I. B., Ye, X., Li, H., & Wilton, J. W. (2006). Association of a single nucleotide polymorphism in the calpastatin gene with carcass and meat quality traits of beef cattle. *Journal of Animal Science, 84*(2), 291-299.

Schnebel, E. M., & Grossfield, J. (1988). Antagonistic Pleiotropy: An Interspecific *Drosophila* Comparison. *Evolution, 42*(2), 306-311.

Selman, C., & Withers, D. J. (2011). Mammalian models of extended healthy life-span. *Philosophical Transactions of the Royal Society B: Biological Sciences, 366*(1561), 99-107. doi:10.1098/rstb.2010.0243

Sepp-Lorenzino, L. (1998). Structure and function of the insulin-like growth factor I receptor. *Breast Cancer Research and Treatment, 47*(3), 235-253. doi:10.1023/a:1005955017615

Sewalem, A., Kistemaker, G. J., Miglior, F., & Van Doormaal, B. J. (2006). Analysis of inbreeding and its relationship with functional longevity in Canadian dairy cattle. *Journal of Dairy Science, 89*(6), 2210-2216.

Sgrò, C. M., & Partridge, L. (1999). A delayed wave of death from reproduction in *Drosophila*. *Science, 286*(5449), 2521-2524. doi:10.1126/science.286.5449.2521

Sharp, P. M., Averof, M., Lloyd, A. T., Matassi, G., & Peden, J. F. (1995). DNA sequence evolution: the sounds of silence. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences, 349*(1329), 241-247. doi:10.1098/rstb.1995.0108

Shawi, M., & Autexier, C. (2008). Telomerase, senescence and ageing. *Mechanisms of Ageing and Development, 129*(1-2), 3-10.

Shenghui, H., Nakada, D., & Morrison, S. J. (2009). Mechanisms of stem cell self-renewal. *Annual Review of Cell and Developmental Biology, 25*(1), 377-406. doi:10.1146/annurev.cellbio.042308.113248

Shimada, M., Yanai, Y., Okazaki, T., Noma, N., Kawashima, I., Mori, T., & Richards, J. S. (2008). Hyaluronan fragments generated by sperm-secreted hyaluronidase stimulate cytokine/chemokine production via the TLR2 and TLR4 pathway in cumulus cells of ovulated COCs, which may enhance fertilization. *Development, 135*(11), 2001-2011. doi:10.1242/dev.020461

Simmons, P., & Ekarius, C. (Eds.). (2001). *Storey's guide to raising sheep*: North Adams, MA: Storey Publishing LLC. ISBN 9781580172622.

Simon, M. D. P. D. K., & Johns, M. D. R. (1999). Mitochondrial disorders: clinical and genetic features. *Annual Review of Medicine, 50*(1), 111-127. doi:doi:10.1146/annurev.med.50.1.111

Sjögren, K., Wallenius, K., Liu, J.-L., Bohlooly-Y, M., Pacini, G., Svensson, L., . . . Ohlsson, C. (2001). Liver-derived IGF-I is of importance for normal carbohydrate and lipid metabolism. *Diabetes, 50*(7), 1539-1545. doi:10.2337/diabetes.50.7.1539

Slijepcevic, P. (2008). DNA damage response, telomere maintenance and ageing in light of the integrative model. *Mechanisms of Ageing and Development, 129*(1-2), 11-16.

Smirnova, I., Hamblin, M. T., McBride, C., Beutler, B., & Di Rienzo, A. (2001). Excess of rare amino acid polymorphisms in the toll-like receptor 4 in humans. *Genetics, 158*(4), 1657-1664.

Smirnova, I., Poltorak, A., Chan, E. K. L., McBride, C., & Beutler, B. (2000). Phylogenetic variation and polymorphism at the Toll-like receptor 4 locus (TLR4). *Genome Biology, 1*(1), research002.001 - research002.010.

Soboll, G., Schaefer, T. M., & Wira, C. R. (2006). Effect of toll-like receptor (TLR) agonists on TLR and microbicide expression in uterine and vaginal tissues of the mouse. *American Journal of Reproductive Immunology, 55*(6), 434-446. doi:10.1111/j.1600-0897.2006.00381.x

Sorimachi, H., Hata, S., & Ono, Y. (2011). Impact of genetic insights into calpain biology. *Journal of Biochemistry, 150*(1), 23-37. doi:10.1093/jb/mvr070

Sorimachi, H., Ishiura, S., & Suzuki, K. (1997). Structure and physiological function of calpains. *The Biochemical Journal, 328(Pt3)*, 721-732.

Soto-Vega, E., Richaud-Patin, Y., & Llorente, L. (2005). Human leukocyte antigen class I, class II, and tumor necrosis factor-alpha polymorphisms in a healthy elder Mexican Mestizo population. *Immunity & Ageing, 2*(1), 13.

Squìer, M. K., Miller, A. C., Malkinson, A. M., & Cohen, J. J. (1994). Calpain activation in apoptosis. *Journal of Cellular Physiology, 159(2)*, 229-237.

Suh, Y., Atzmon, G., Cho, M.-O., Hwang, D., Liu, B., Leahy, D. J., . . . Cohen, P. (2008). Functionally significant insulin-like growth factor I receptor mutations in centenarians. *Proceedings of the National Academy of Sciences of the United States of America, 105*(9), 3438-3442. doi:10.1073/pnas.0705467105

Taguchi, A., & White, M. F. (2008). Insulin-like signaling, nutrient homeostasis, and life-span. *Annual Review of Physiology, 70*(1), 191-212. doi:10.1146/annurev.physiol.70.113006.100533

Taioli, E., Mari, D., Franceschi, C., Bonafè, M., Monti, D., Bertolini, S., . . . Garte, S. (2001). Polymorphisms of drug-metabolizing enzymes in healthy nonagenarians and centenarians: difference at GSTT1 locus. *Biochemical and Biophysical Research Communications, 280*(5), 1389-1392.

Takahashi, Y., Kuro-o, M., & Ishikawa, F. (2000). Aging mechanisms. *Proceedings of the National Academy of Sciences of the United States of America, 97*(23), 12407-12408. doi:10.1073/pnas.210382097

Takano, J., Mihira, N., Fujioka, R., Hosoki, E., Chishti, A. H., & Saido, T. C. (2011). Vital role of the calpain-calpastatin system for placental-integrity-dependent embryonic survival. *Molecular and Cellular Biology, 31*(19), 4097-4106. doi:10.1128/mcb.05189-11

Takano, J., Watanabe, M., Hitomi, K., & Maki, M. (2000). Four types of calpastatin isoforms with distinct amino-terminal sequences are specified by alternative first exons and differentially expressed in mouse tissues. *Journal of Biochemistry, 128(1)*, 83-92.

Takeda, K., Kaisho, T., & Akira, S. (2003). Toll-like receptors. *Annual Review of Immunology, 21*, 335-376.

Tan, Q., Yashin, A. I., Bladbjerg, E. M., de Maat, M. P. M., Andersen-Ranberg, K., Jeune, B., . . . Vaupel, J. W. (2001). Variations of cardiovascular disease associated genes exhibit sex-dependent influence on human longevity. *Experimental Gerontology, 36*(8), 1303-1315.

Tatar, M., Bartke, A., & Antebi, A. (2003). The endocrine regulation of aging by insulin-like signals. *Science, 299*(5611), 1346-1351. doi:10.1126/science.1081447

Taylor, D., Maxwell, M., Luthi-Carter, R., & Kazantsev, A. (2008). Biological and potential therapeutic roles of sirtuin deacetylases. *Cellular and Molecular Life Sciences, 65*(24), 4000-4018. doi:10.1007/s00018-008-8357-y

Taylor, J. P., Hardy, J., & Fischbeck, K. H. (2002). Toxic proteins in neurodegenerative disease. *Science, 296*(5575), 1991-1995. doi:10.1126/science.1067122

Teixeira, I. N. D. A. O., & Guariento, M. E. (2010). Biologia do envelhecimento: teorias, mecanismos e perspectivas. *Ciência & Saúde Coletiva, 15*, 2845-2857.

Thakore, J. H., & Dinan, T. G. (1994). Growth hormone secretion: the role of glucocorticoids. *Life Sciences, 55(14)*, 1083-1099.

Thompson, V. F., Saldana, S., Cong, J., Luedke, D. M., & Goll, D. E. (2002). The calpain system in human placenta. *Life Sciences, 70(21)*, 2493-2508.

Tran, H., Brunet, A., Grenier, J. M., Datta, S. R., Fornace, A. J., DiStefano, P. S., . . . Greenberg, M. E. (2002). DNA repair pathway stimulated by the forkhead transcription factor FOXO3a through the Gadd45 protein. *Science, 296(5567)*, 530-534.

Trifunovic, A., & Larsson, N. G. (2008). Mitochondrial dysfunction as a cause of ageing. *Journal of Internal Medicine, 263*(2), 167-178. doi:10.1111/j.1365-2796.2007.01905.x

Trinchieri, G., & Sher, A. (2007). Cooperation of Toll-like receptor signals in innate immune defence. *Nature Reviews Immunology, 7*(3), 179-190.

Tsai, K.-L., Sun, Y.-J., Huang, C.-Y., Yang, J.-Y., Hung, M.-C., & Hsiao, C.-D. (2007). Crystal structure of the human FOXO3a-DBD/DNA complex suggests the effects of post-translational modification. *Nucleic Acids Research, 35*(20), 6984-6994. doi:10.1093/nar/gkm703

Tsan, M.-F., & Gao, B. (2004). Endogenous ligands of Toll-like receptors. *Journal of Leukocyte Biology, 76*(3), 514-519. doi:10.1189/jlb.0304127

Tullet, J. M., Hertweck, M., An, J. H., Baker, J., Hwang, J. Y., Liu, S., . . . Blackwell, T. K. (2008). Direct inhibition of the longevity-promoting factor SKN-1 by insulin-like signaling in *C. elegans*. *Cell, 132*(6), 1025-1038.

Turkalj, I., Keller, U., Ninnis, R., Vosmeer, S., & Stauffacher, W. (1992). Effect of increasing doses of recombinant human insulin-like growth factor-I on glucose, lipid, and leucine metabolism in man. *Journal of Clinical Endocrinology & Metabolism, 75*(5), 1186-1191. doi:10.1210/jc.75.5.1186

Turturro, A., Blank, K., Murasko, D., & Hart, R. (1994). Mechanisms of caloric restriction affecting aging and disease. *Annals of the New York Academy of Sciences, 719*(1), 159-170. doi:10.1111/j.1749-6632.1994.tb56827.x

Uematsu, S., & Akira, S. (2006). The role of Toll-like receptors in immune disorders. *Expert Opinion on Biological Therapy, 6*(3), 203-214. doi:doi:10.1517/14712598.6.3.203

Uematsu, S., & Akira, S. (2008). Toll-like receptors (TLRs) and their ligands. In (183 ed., pp. 1-20): Springer Berlin Heidelberg. Retrieved from http://dx.doi.org/10.1007/978-3-540-72167-3_1. doi:10.1007/978-3-540-72167-3_1

Vaisid, T., Kosower, N. S., Katzav, A., Chapman, J., & Barnoy, S. (2007). Calpastatin levels affect calpain activation and calpain proteolytic activity in APP transgenic mouse model of Alzheimer's disease. *Neurochemistry International, 51*(6–7), 391-397.

van den Hemel-Grooten, H. N., te Pas, M. F., van den Bosch, T. J., Garssen, G. J., Schreurs, V. V., & Verstegen, M. W. (1997). mRNA levels of the calpain system in longissimus muscle of young pigs during prolonged feeding of a protein-free diet. *Journal of Animal Science, 75*(4), 968-974.

van der Heide, L. P., Hoekman, M. F., & Smidt, M. P. (2004). The ins and outs of FOXO shuttling: mechanisms of FOXO translocation and transcriptional regulation. *The Biochemical Journal, 380(Pt2)*, 297-309.

van der Heide, L. P., & Smidt, M. P. (2005). Regulation of FoxO activity by CBP/p300-mediated acetylation. *Trends in Biochemical Sciences, 30*(2), 81-86.

van der Vos, K. E., & Coffer, P. J. (2008). FOXO-binding partners: it takes two to tango. *Oncogene, 27*(16), 2289-2299.

Van der Werf, J. H. J. (Ed.). (2007). *Marker-assisted selection, Marker-assisted selection in sheep and goat (Chap.13)*. Rome: Viale delle Terme di Caracalla.

Van Duin, D., & Shaw, A. C. (2007). Toll-like receptors in older adults. *Journal of the American Geriatrics Society, 55*(9), 1438-1444. doi:10.1111/j.1532-5415.2007.01300.x

Van Heemst, D., Beekman, M., Mooijaart, S. P., Heijmans, B. T., Brandt, B. W., Zwaan, B. J., . . . Westendorp, R. G. J. (2005). Reduced insulin/IGF-1 signalling and human longevity. *Aging Cell, 4*(2), 79-85. doi:10.1111/j.1474-9728.2005.00148.x

VanRaden, P. M., & Klaaskate, E. J. H. (1993). Genetic evaluation of length of productive life including predicted longevity of live cows. *Journal of Dairy Science, 76*(9), 2758-2764.

Varani, S., & Matzuk, M. M. (2002). Phenotypic effects of knockout of oocyte-specific genes. *Ernst Schering Research Foundation Workshop, 41*, 63-79.

Vasto, S., Candore, G., Balistreri, C. R., Caruso, M., Colonna-Romano, G., Grimaldi, M. P., . . . Caruso, C. (2007). Inflammatory networks in ageing, age-related diseases and longevity. *Mechanisms of Ageing and Development, 128*(1), 83-91.

Vasto, S., & Caruso, C. (2004). Immunity & Ageing: a new journal looking at ageing from an immunological point of view. *Immunity & Ageing, 1*(1), 1.

Vellai, T. (2009). Autophagy genes and ageing. *Cell Death and Differentiation, 16*(1), 94-102.

Vermeulen, K., Van Bockstaele, D. R., & Berneman, Z. N. (2003). The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. *Cell Proliferation, 36*(3), 131-149. doi:10.1046/j.1365-2184.2003.00266.x

Vijg, J., & Suh, Y. (2004). Genetics of longevity and aging. *Annual Review of Medicine, 56*(1), 193-212. doi:10.1146/annurev.med.56.082103.104617

Voland, E., & Engel, C. (1989). Women's reproduction and longevity in a premodern population (Ostfriesland, Germany, 18th century). In *The Sociobiology of Sexual and Reproductive Strategies (ed. A.E. Rasa, C. Vogel & Voland)* (pp. 194-205): London: Chapman & Hall.

Walker, G., Houthoofd, K., Vanfleteren, J. R., & Gems, D. (2005). Dietary restriction in *C. elegans*: From rate-of-living effects to nutrient sensing pathways. *Mechanisms of Ageing and Development, 126*(9), 929-937.

Walker, J. L., Van Wyk, J. J., & Underwood, L. E. (1992). Stimulation of statural growth by recombinant insulin-like growth factor 1 in a child with growth hormone insensitivity syndrome (Laron type). *The Journal of Pediatrics, 121(4)*, 641-646.

Wall, E., Brotherstone, S., & Coffey, M. P. (2006). In '8th World Congress on Genetics Applied to Animal Production'. In *Belo, Horizonte, Minas Gerais, Brazil* (pp. 1-10)

Wang, B., Mu, Y., Ni, F., Zhou, S., Wang, J., Cao, Y., & Ma, X. (2010). Analysis of FOXO3 mutation in 114 Chinese women with premature ovarian failure. *Reproductive BioMedicine Online, 20*(4), 499-503.

Wang, F., Marshall, C. B., Yamamoto, K., Li, G.-Y., Plevin, M. J., You, H., . . . Ikura, M. (2008). Biochemical and structural characterization of an intramolecular interaction in FOXO3a and its binding with p53. *Journal of Molecular Biology, 384*(3), 590-603.

Wang, J. I. E., Zhou, J., & Bondy, C. A. (1999). Igf1 promotes longitudinal bone growth by insulin-like actions augmenting chondrocyte hypertrophy. *The FASEB Journal, 13*(14), 1985-1990.

Wang, K. K., & Yuen, P. W. (1994). Calpain inhibition: an overview of its therapeutic potential. *Trends in Phamacological Sciences, 15(11)*, 412-419.

Wang, M., Zhang, X., Zhao, H., Wang, Q., & Pan, Y. (2009). FoxO gene family evolution in vertebrates. *BMC Evolutionary Biology, 9*(1), 222.

Watkins, W. J., Umbers, A. J., Woad, K. J., Harris, S. E., Winship, I. M., Gersak, K., & Shelling, A. N. (2006). Mutational screening of FOXO3A and FOXO1A in women with premature ovarian failure. *Fertility and Sterility, 86*(5), 1518-1521.

Wauters, L., & Dhondt, A. A. (1989). Body weight, longevity and reproductive success in red squirrels (*Sciurus vulgaris*). *Journal of Animal Ecology, 58*(2), 637-651.

Weindruch, R. (1996). The retardation of aging by caloric restriction: studies in rodents and primates. *Toxicologic Pathology, 24*(6), 742-745. doi:10.1177/019262339602400618

Weinert, B. T., & Timiras, P. S. (2003). Invited review: Theories of aging. *Journal of Applied Physiology, 95*(4), 1706-1716. doi:10.1152/japplphysiol.00288.2003

Werling, D., & Jungi, T. W. (2003). Toll-like receptors linking innate and adaptive immune response. *Veterinary Immunology and Immunopathology, 91*(1), 1-12.

Westendorp, R. G. J., & Kirkwood, T. B. L. (1998). Human longevity at the cost of reproductive success. *Nature, 396*(6713), 743-746.

Wherry, E. J., & Ahmed, R. (2004). Memory CD8 T-Cell Differentiation during Viral Infection. *Journal of Virology, 78*(11), 5535-5545. doi:10.1128/jvi.78.11.5535-5545.2004

White, S. N., Taylor, K. H., Abbey, C. A., Gill, C. A., & Womack, J. E. (2003). Haplotype variation in bovine Toll-like receptor 4 and computational prediction of a positively selected ligand-binding domain. *Proceedings of the National Academy of Sciences of the United States of America, 100*(18), 10364-10369. doi:10.1073/pnas.1333957100

Willcox, B. J., Donlon, T. A., He, Q., Chen, R., Grove, J. S., Yano, K., . . . Curb, J. D. (2008). FOXO3A genotype is strongly associated with human longevity. *Proceedings of the National Academy of Sciences of the United States of America, 105*(37), 13987-13992. doi:10.1073/pnas.0801030105

Williams, J. L. (2005). The use of marker-assisted selection in animal breeding and biotechnology. *Revue Scientificque et Technique, 24*, 379-391.

Wright, J., & Hausman, G. (1995). Insulinlike growth factor-1 (IGF-1)-induced stimulation of porcine preadipocyte replication. *In Vitro Cellular & Developmental Biology - Animal, 31*(5), 404-408. doi:10.1007/bf02634290

Wu, G., Bazer, F. W., Wallace, J. M., & Spencer, T. E. (2006). Board-invited review: Intrauterine growth retardation: Implications for the animal sciences. *Journal of Animal Science, 84*(9), 2316-2337. doi:10.2527/jas.2006-156

Xuan, Z., & Zhang, M. Q. (2005). From worm to human: bioinformatics approaches to identify FOXO target genes. *Mechanisms of Ageing and Development, 126*(1), 209-215.

Yajima, Y., Sato, M., Sorimachi, H., Inomata, M., Maki, M., & Kawashima, S. (2006). Calpain system regulates the differentiation of adult primitive mesenchymal ST-13 adipocytes. *Endocrinology, 147*(10), 4811-4819. doi:10.1210/en.2005-1647

Yamashita, S., & Melmed, S. (1986). Insulin-like growth factor I action on rat anterior pituitary cells: suppression of growth hormone secretion and messenger ribonucleic acid levels. *Endocrinology, 118*(1), 176-182. doi:10.1210/endo-118-1-176

Yamaya, M., Nakayama, K., Ebihara, S., Hirai, H., Higuchi, S., & Sasaki, H. (2003). Relationship between microsatellite polymorphism in the haem oxygenase-1 gene promoter and longevity of the normal Japanese population. *Journal of Medical Genetics, 40*(2), 146-148. doi:10.1136/jmg.40.2.146

Yanagimachi, R., & Bhattacharyya, A. (1988). Acrosome-reacted guinea pig spermatozoa become fusion competent in the presence of extracellular potassium ions. *The Journal of Experimental Zoology, 248(3)*, 354-360.

Youngson, N., Vickaryous, N., van der Horst, A., Epp, T., Harten, S., Fleming, J., . . . Whitelaw, E. (2011). A missense mutation in the transcription factor Foxo3a causes teratomas and oocyte abnormalities in mice. *Mammalian Genome, 22*(3), 235-248. doi:10.1007/s00335-011-9317-7

Zatz, M., & Starling, A. (2005). Calpains and disease. *New England Journal of Medicine, 352*(23), 2413-2423. doi:doi:10.1056/NEJMra043361

Zeytun, A., van Velkinburgh, J. C., Pardington, P. E., Cary, R. R., & Gupta, G. (2007). Pathogen-specific innate immune response. *Advances in Experimental Medicine and Biology, 598*, 342-357.

Zhou, B. B., & Elledge, S. J. (2000). The DNA damage response: putting checkpoints in perspective. *Nature, 408*(6811), 433-439.

Zhou, H., Hickford, J. G. H., & Fang, Q. (2006). A two-step procedure for extracting genomic DNA from dried blood spots on filter paper for polymerase chain reaction amplification. *Analytical Biochemistry, 354*(1), 159-161.

Zhou, H., Hickford, J. G. H., Fang, Q., & Lin, Y.-S. (2007b). Allelic variation of the ovine Toll-like receptor 4 gene. *Developmental & Comparative Immunology, 31*(2), 105-108.

Zhou, H., Hickford, J. G. H., & Gong, H. (2007a). Polymorphism of the ovine calpastatin gene. *Molecular and Cellular Probes, 21*(3), 242-244.

Zhu, W., Bijur, G. N., Styles, N. A., & Li, X. (2004). Regulation of FOXO3a by brain-derived neurotrophic factor in differentiated human SH-SY5Y neuroblastoma cells. *Molecular Brain Research, 126*(1), 45-56.

References for appendix A and B

Belt, P.B.G.M., Muileman, I.H., Schreuder, B.E.C., Bosderuijter, J., Gielkens, A.L.J., & Smits, M.A. (1995). Identification of five allelic variants of the sheep Prp gene and their association with natural scrapie, *Journal of General Virology, 76*, 509-517.

Berg, T., Healy, P.J., Tollersrud, O.K., & Nilssen, O. (1997). Molecular heterogeneity for bovine alpha-mannosidosis-PCR based assays for detection of breed-specific mutations. *Research in Veterinary Science, 63*, 279-282.

Bodin, L., Di Pasquale, E., Fabre, S., Bontoux, M., Monget, P., Persani, L. & Mulsant, P. (2007). A novel mutation in the bone morphogenetic protein 15 gene causing defective protein secretion is associated with both increased ovulations rate and sterility in Lacaune sheep. *Endocrinology, 148*, 393-400.

Borchersen, S., (2001). Danish scientists reveal the gene responsible for CVM, a lethal heritable defect in Holstein Cattle. Danish Cattle Breeding, press release 2001 08 17. Available: http://www.lr.dk/kvaeg/diverse/PRESS-uk.htm.Accessed Feb. 9, 2004.

Chu, M.X., Liu, Z.H., Jiao, C.L., He, Y.Q., Fang, L., Ye, S.C., Chen, G.H., & Wang, J.Y. (2007). Mutations in *BMPR-IB* and *BMP-15* genes are associated with litter size in small tailed Han sheep (*Ovis aries*). *Journal of Animal Science, 85*, 598-603.

Ciobanu, D., Bastiaansen, J., Malek, M., Helm, J., Plastow, G., Woollard, J., & Rothschild, M. (2001). Evidence for new alleles in the protein kinase adenosine monophosphate activated gamma3-subunit gene associated with low glycogen content in pig skeletal muscle and improved meat quality. *Genetics, 158*, 1151-1162.

Ciobanu, D.C., Bastiaansen, J.W., Lonergan, S.M., Thomsen, H., Dekkers, J.C., Plastow, G.S., & Rothschild, M.F. (2004). New alleles in calpastatin gene are associated with meat quality traits in pigs. *Journal of Animal Science. 82*, 2829-2839.

Dekkers, J.C. (2004). Commercial application of marker-and gene-assisted selection in livestock: strategies and lessons. *Journal of Animal Science, 82*, E313-E328.

Dennis, J.A., & Healy, P.J. (1999). Definition of the mutation responsible for maple syrup urine disease in Poll Shorthorns and genotyping Poll Shorthorns and Poll Herefords for maple syrup urine disease alleles. *Research in Veterinary Science, 67*, 1-6.

Dennis, J.A., Healy, P.J., Beaudet, A.L., & Obrien, W.E. (1989). Molecular definition of bovine argininosuccinate synthetise deficiency. *Proceedings of the national academy of sciences of the United States of America*, *86*, 7947-7951.

Forrest, R.H., Hickford, J.G., & Frampton, C.M. (2007). Polymorphism at the ovine beta3-adrenergic receptor locus (ADRB3) and its association with lamb mortality. *Journal of Animal Science, 85*, 2801-2806.

Freking, B., Murphy, S.K., Wylie, A.A., Rhodes, S.J., Keele, J.W., Leymaster, K.A. Jirtle, R.L. & Smith, T.P. (2002). Identification of the single base change causing the callipyge muscle hypertrophy phenotype, the only known example of polar overdominance in mammals. *Genome Research, 10*, 1496-1506.

Fuji, J., Otsu, K., Zorzato, F., De Leon, S., Khanna, V.K., Weiler, J.E., O'Brien, P.J., & Maclennan, D.H. (1991). Identification of a mutation in porcine ryanodine receptor associated with malignant hyperthermia. *Science, 253*, 448-451.

Galloway, S.M., McNatty, K.P., Cambridge, L.M., Laitinen, M.P.E., Juengel, J.L., Jokiranta, T.S., McLaren, R.J., Luiro, K., Dodds, K.G., Montgomery, G.W., Beattie, A.E., Davis, G.H., & Ritvos, O. (2000). Mutations in an oocyte-derived growth factor gene (BMP15) cause increased ovulation rate and infertility in a dosage-sensitive manner. *Nature Genetics, 25*, 279-283.

Grobet, L., Poncelet, D., Royo, L.J., Brouwers, B., Pirottin, D., Michaux, C., Menissier, F., Zanotti, M., Dunner, S., & Georges, M. (1998). Molecular definition of an allelic

series of mutations disrupting the myostatin function and causing double-muscling in cattle. *Mammalian Genome, 9*, 210-213.

Hanrahan, J.P., Gregan, S.M., Mulsant, P., Mullen, M., Davis, G.H., Powell, R., & Galloway, S.M. (2004). Mutations in the genes for oocyte-derived growth factors GDF9 and BMP15 are associated with both increased ovulation rate and sterility in Cambridge and Belclare sheep (*Ovis aries*). *Biology of Reproduction, 70*, 900-909.

Hickford, J.G., Zhou, H., Slow, S., & Fang, Q. (2004). Diversity of the ovine DQA2 gene. *Journal of animal Science. 82*, 1553-1563.

Hickford, J.G., Zhou, H., Fang, Q., Byun, S.O., & Gong, H. (2008). Frequency of PRNP genotypes in common New Zealand sheep breed. The Veterinary Record, 163, 453-454.

Houston, R.D., Rance, K.A., Sutcliffe, E., Archibald, A.L. & Haley, C.S. (2008). The cholecystokinin type A receptor g.179A>G polymorphism affects feeding rate. Animal Genetics, 39, 187-188.

Jeon, J., Carlborg, O., Tornsten, A., Giuffra, E., Amarger, V., Chardon, P., Andersson-Eklund, L., & Andersson, L. (1999). A paternally expressed QTL affecting skeletal and cardiac muscle mass in pigs maps to the IGF2 locus. *Nature Genetics, 21*, 157-158.

Khatib, H., Monson, R.L., Schutzkus, V., Kohl, D.M., Rosa, G.J., & Rutledge, J.J. (2008). Mutations in the STAT5A gene are associated with embryonic survival and milk composition in cattle. *Journal of Dairy Science, 91*, 784-793.

Kim, K.S., Larsen, N., Short, T., Plastow, G., & Rothschild, M.F. (2000). A missense variant of the porcine melanocortin-4-receptor (MC4R) gene is associated with fatness, growth and feed intake traits, *Mammalian Genome, 11*, 131-135.

Lunden, A., Marklund, S., Gustafsson, V., & Andersson, L. (2002). A nonsense mutation in the FMO3 gene underlies fishy off-flavour in cow's milk. *Genome Research, 12*, 1885-1888.

Marklund, S., Kijas, J., Rodriguezmartinez, H., Ronnstrand, L., Funa, K., Moller, M., Lange, D., Edforslilja, I., & Andersson, L. (1998). Molecular basis for the dominant white phenotype in the domestic pig. *Genome Research, 8*, 826-833.

McNatty, K.P., Jeungel, J.L., Wilson, T., Calloway, S.M., & Davis, G.H. (2001). Genetic mutations influencing ovulation rate in sheep. *Reproduction, Fertility and Development, 13*, 549-555.

Medrano, J.F., & Aquilar-Cordova, E. (1990). Polymerase chain reaction amplification of bovine β-lactoglobulin genomic sequences and identification of genetic variants by RFLP analysis. *Animal Biotechnology, 1*, 73-77.

Mulsant, P., Lecerf, F., Fabre, S., Schibler, L., Monget, P., Lanneluc, I., Pisselet, C., Riguet, J., Monniaux, D., Callebaut, I., Cribiu, E., Thimonier, J., Teyssier, J., Bodin, L., Cognié, Y., Chitour, N., & Elsen, J.M. (2001). Mutation in bone morphogenetic protein receptor-IB is associated with increased ovulations rate in Booroola Merino ewes. *Proceedings of the national academy of sciences of the United States of America*, *98*, 5104-5109.

Tomás, A., Casellas, J., Ramírez, O., Muñoz, G., Noguera, J.L., & Sánchez, A. (2006). High amino acid variation in the intracellular domain of the pig prolactin receptor (PRLR) and its relation to ovulation rate and piglet survival traits. *Journal of animal Science, 84*, 1991-1998.

Muñoz, G., Ovilo, C., Estellé, J., Silió, L., Fernández, A., & Rodriguez, C. (2007). Association with litter size of new polymorphisms on *ESR1* and *ESR2* genes in a Chinese-European pig line. *Genetics, Selection, Evolution, 39*, 195, 206.

Schwenger, B., Schober, S., & Simon, D. (1993). DUMPS cattle carry a point mutation in the uridine monophosphate synthase gene. *Genomics, 16*, 241-244.

Shuster, D.E., Kehrli, M.E., Ackermann, M.R., & Gilbert, R.O. (1992). Identification and prevalence of a genetic defect that causes leukocyte adhesion deficiency in Holstein cattle. *Proceedings of the national academy of sciences of the United States of America*, *89*, 9225-9229.

van Eenennaam, A.L., Li, J., Thallman, R.M., Quaas, R.L., Dikeman, M.E., Gill, C.A., Franke, D.E., & Thomas, M.G. (2007). Validation of commercial DNA tests for quantitative beef quality traits. *Journal of Animal Science*, *85*, 891-900.

Wilson, T., Wu, X.Y., Juengel., J.L., Ross, I.K., Lumsden, J.M., Lord, E.A., Dodds, K.G., Walling, G.A., McEwan, J.C., O'Connell, A.R., McNatty, K.P., & Montgomery, G.W. (2001). Highly prolific Booroola sheep have a mutation in the intracellular kinase domain of bone morphogenetic protein IB receptor (ALK-6) that is expressed in both oocytes and granulose cells. *Biology of Reproduction, 64*, 1225-1235.

Appendices

Appendix A: Examples of DNA tests used in improving production and disease resistance traits in livestock

Trait category	Species	Gene Marker	Reference
Congenital defects	Dairy Cattle	BLAD	Shuster et al. (1992)
	Cattle	Citrulinaemia	Dennis et al. (1989)
	Dairy Cattle	DUMPS	Schwenger et al. (1993)
	Dairy Cattle	CVM	Borchersen (2001)
	Cattle	Maple syrup urine	Dennis & Healy (1999)
	Pig	RYR	Fuji et al. (1991)
Appearance	Pig	CKIT	Marklund et al. (1998)
	Cattle	Red coat colour	Dekker (2004)
	Cattle	Black coat colour	Van Eenemmaam et al. (2007)
	Cattle/Pig	MC1R/MSHR	
	Beef Cattle	MGF	
Genetic disorder	Cattle	Mannosidosis	Berg et al. (1997)
		Dwarfism	Van Eenemmaam et al. (2007)
		Weaver syndrome	
		Platelet bleeding	
		Albinism	
Milk quality	Dairy Cattle	κ-Casein	Medrano & Aquilar-Cordova (1990)
		β-lactoglobulin	
		FMO3	Lunden et al. (2002)
Feed intake	Pig	MC4R	Kim et al. (2000)
Reproduction	Sheep	Booroola	Wilson et al. (2001)
		Inverdale	Galloway et al. (2000)
		Hanna	McNatty et al. (2001)
Growth and Composition	Pig	MC4R	Kim et al. (2000)
	Pig	IGF-2	Jeon et al. (1999)
	Beef Cattle	Myostatin	Grobet et al. (1998)
	Sheep	Callipyge	Freking et al. (2002)
	Pig	CAST	Ciobanu et al. (2004)
Meat quality	Cattle/Pig	CAST	Ciobanu et al. (2004)
		CAPN1	Van Eenemmaam et al. (2007)
		CAPN2	
		RN/PRKAG3	Ciobanu et al. (2001)
Disease resistance	Sheep	Footrot	Hickford et al. (2004)
		Scrapie	Hickford et al. (2008)
		Prp	Belt et al. (1995)
		Cold tolerance	Forrest et al. (2007)

Appendix B: DNA variation and effects on reproduction traits in cattle, goat, pig and sheep

Gene	Species	DNA variation	Effect on trait	Reference
ADIPOQ (Adiponectin)	Pig	c.178G>A	Fewer stillborn piglets, shorter weaning-to-oestrus intervals	Houde et al. (2008)
ADIPOR1 (Adiponectin receptor 1)	Pig	c.129A>C	Liveborn piglets	Houde et al. (2008)
ADIPOR2 (Adiponectin receptor 2)	Pig	c.112G>A	Weaning-to-oestrus intervals	Houde et al. (2008)
BMPR-IB (Bone morphogenetic protein receptor IB)	Sheep	*FecB* mutation, A746G (Gln249Arg)	Hyperprolific trait in Booroola Merino	Mulsant et al. (2001) Wilson et al. (2001)
BMPR-15 (Bone morphogenetic protein receptor - 15)	Sheep	$FecX^G$ mutation, C718T (Gln239stop)	Ovulation rate, sterility	Hanrahan et al. (2004) Chu et al. (2007)
		$FecX^I$ mutation, (Val31Asp)	Inverdale sheep	Galloway et al. (2000)
		$FecX^H$ mutation, (Q23stop)	Hanna sheep	Galloway et al. (2000)
		$FecX^L$ mutation, (Cys53Tyr)	Lacaune sheep	Bodin et al. (2007)
		$FecX^B$ mutation, (Ser99Ile)	Belclare sheep	Hanrahan et al. (2004)
GDF9 (Growth differentiation factor 9)	Sheep	$FecG^H$mutation, (Ser77Phe)	Ovulation rate, sterility in Belclare sheep	Hanrahan et al. (2004)
ESR1 (Estrogen receptor 1)	Pig	c.1227C>T in exon 5	Number of piglets	Muñoz et al. (2007)
FSHβ (Follicle-stimulating hormone β)	Pig	*Bam*HI mutation	Litter size	Li et al. (1998)
PRLR (Prolactin receptor)	Pig	C1217T, C1283A G1439A, T1528A G1600A, G1789A	The number of corpora lutea	Tomas et al. (2006)
STAT (Signal transducer and activator of transcription5A)	Cattle	A3117G (intron 4) C13244T (intron 4) A13244G (intron 9) G12195C (exon 8)	Embryonic survival rate	Khatib et al. (2008)

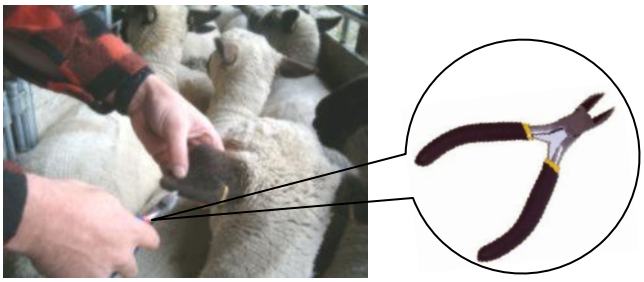
Appendix C: Instruction for Sheep Blood Collection on FTA® Cards

LINCOLN UNIVERSITY
Te Whare Wānaka O Aoraki

Gene-Marker Laboratory
Agriculture & Life Sciences Division
PO Box 84, Lincoln University
Canterbury, NEW ZEALAND
Ph: 0064 3 3253803
0508 FOOTROT
Fax: 0064 3 325 3851

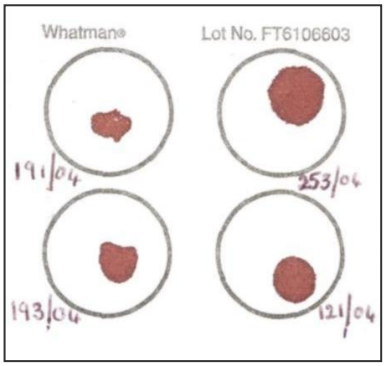
Step 1: Make a small cut about ½ cm into the bottom edge or tip of the ear of the sheep using electrical side-cutters ("diags").

Clean the side-cutters in large amount of water if they are contaminated with blood from previous animals.



Step 2: Collect blood from the cut onto the FTA cards. One card is designed for **FOUR** sheep.

Step 3: Label the FTA cards clearly with a unique ID (e.g. a brass tag number and year) for each blood spot.



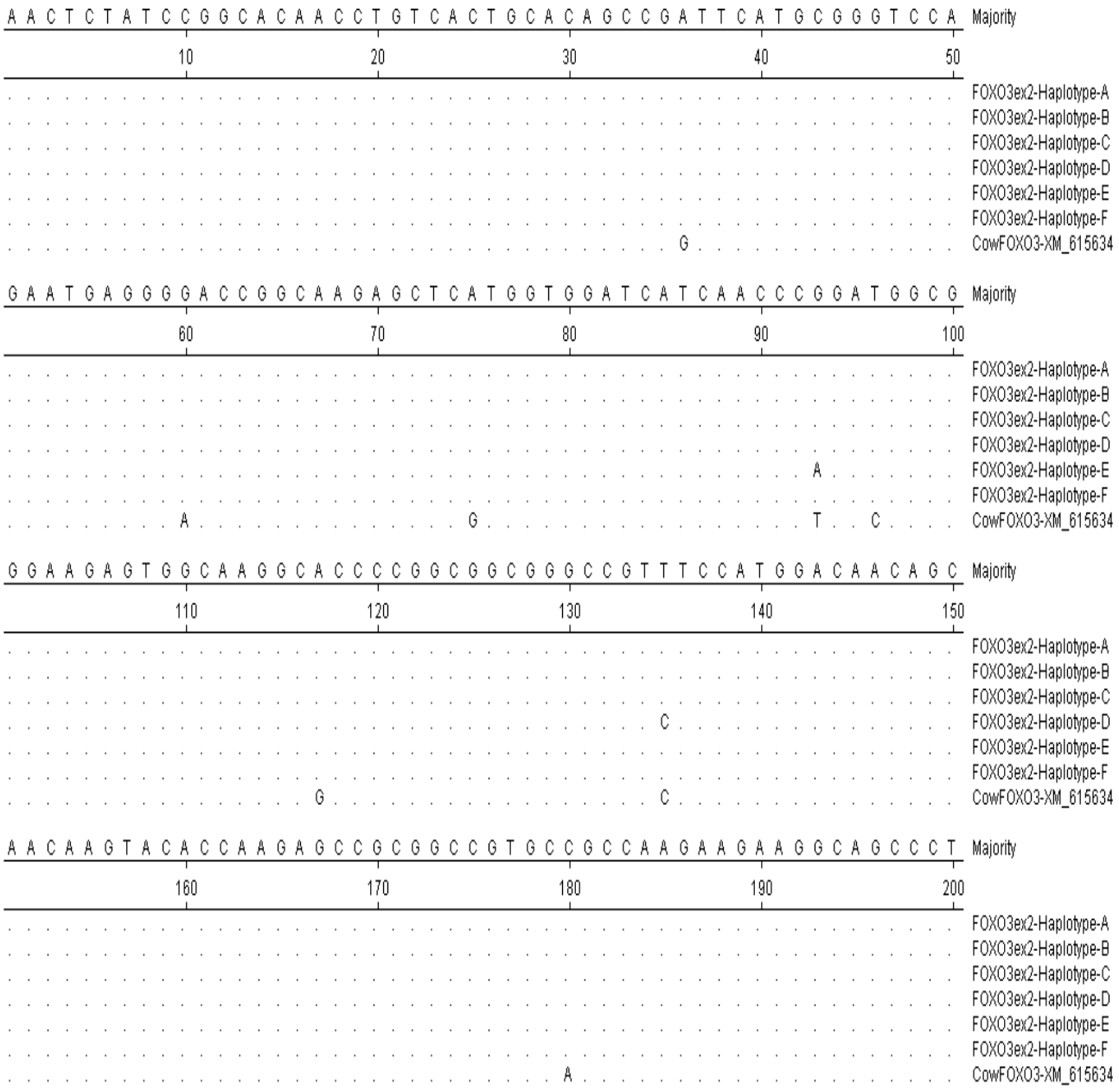
Step 3: Blood samples are air-dried and then can be easily posted (together with a current New Zealand Ministry of Agriculture and Forests Import Permit if from overseas as) to: Gen-Marker Laboratory, Agriculture & Life Science Division

Appendix D: Exon 3 of ovine *IGF1R* (alignment of sequences *A*, *B* and *C* with exon 3 of bovine *IGF1R*)

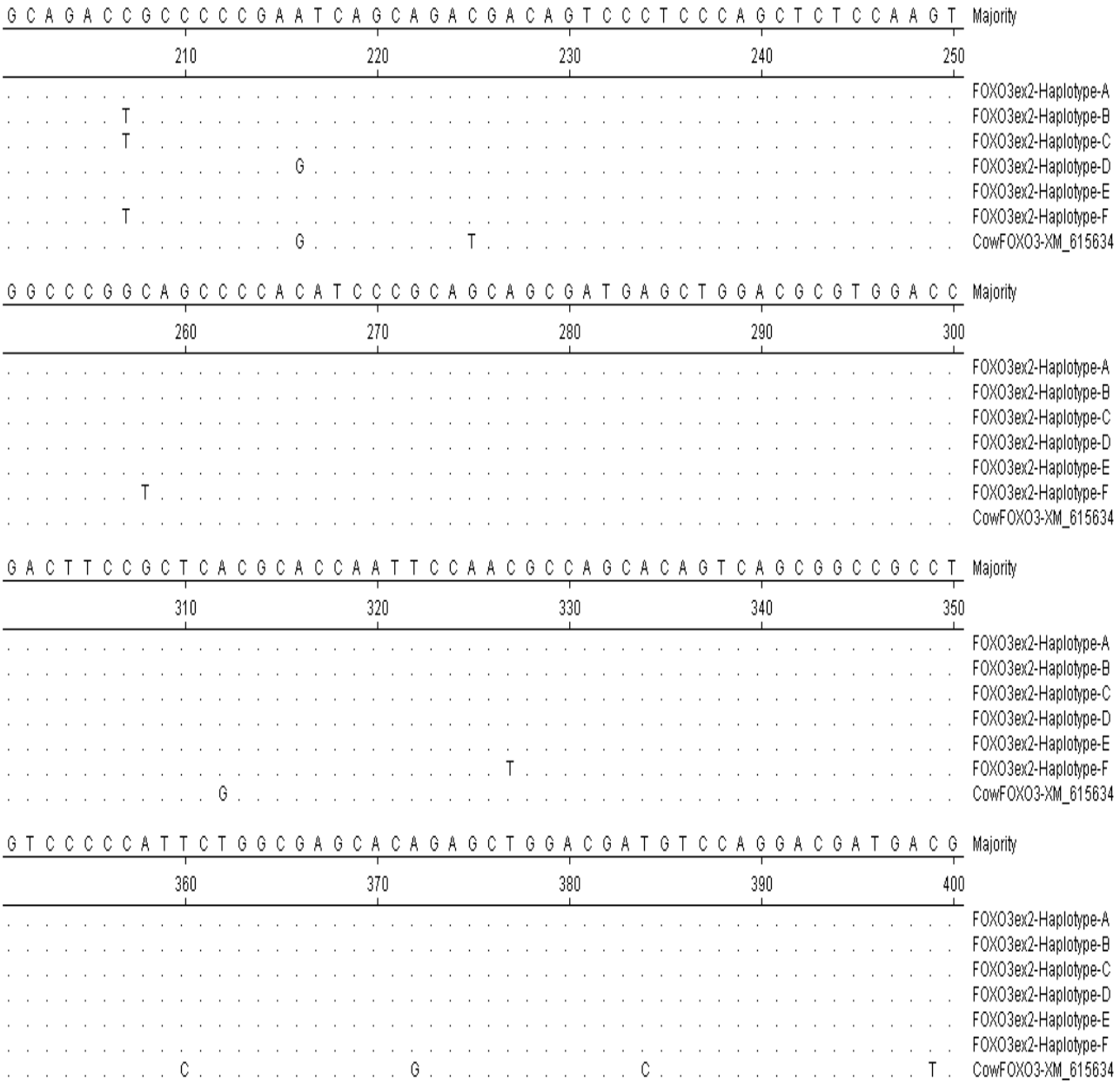
```
IGF1Rex3A    CTCACACCCTGCCTGTCCCCACAGTGTGTCCCAGCGCGTGCGGGAAGCGGGCGTGCACGG 60
IGF1Rex3B    -----------------g---------------------------------------- 60
IGF1Rex3C    ------------------------------------a--------------------- 60
IGF1R-Bovine ----------------------------c-------------------------t---- 60

IGF1Rex3A    AGACCCACGAGTGCTGCCACCCCGAGTGCCTGGGCAGCTGCAGCGCGCCCGACAACGCCA 120
IGF1Rex3B    ------------------------------------------------------------ 120
IGF1Rex3C    ------------------------------------------------------------ 120
IGF1R-Bovine -------t---------------------------------------------------- 120

IGF1Rex3A    CGGCCTGCGTGGCCTGCCGCCACTACTACTACGCCGGCATCTGCGTGCCCAGCTGCCCGC 180
IGF1Rex3B    ------------------------------------------------------------ 180
IGF1Rex3C    ------------------------------------------------------------ 180
IGF1R-Bovine ------------------------------------g---------------------- 180

IGF1Rex3A    CCAACACCTACCGCTTCGAGGGCTGGCGCTGCGTGGACCGCGACTTCTGCGCCAACATCC 240
IGF1Rex3B    ------------------------------------------------------------ 240
IGF1Rex3C    ------------------------------------------------------------ 240
IGF1R-Bovine ------------------------------------------------------------ 240

IGF1Rex3A    CCAACGCCGAGAGCAGCGACTCCGAGGGCTTCGTCATCCACGACGGCGAGTGCATGCAGG 300
IGF1Rex3B    ------------------------------------------------------------ 300
IGF1Rex3C    ------------------------------------------------------------ 300
IGF1R-Bovine ------------------------------------------------------------ 300

IGF1Rex3A    AGTGCCCGTCGGGCTTCATCCGCAACGGGAGCCAGAGGTCAGTGTG 346
IGF1Rex3B    ----------------------------------------------- 346
IGF1Rex3C    ----------------------------------------------- 346
IGF1R-Bovine ----------------------------------------------- 346
```

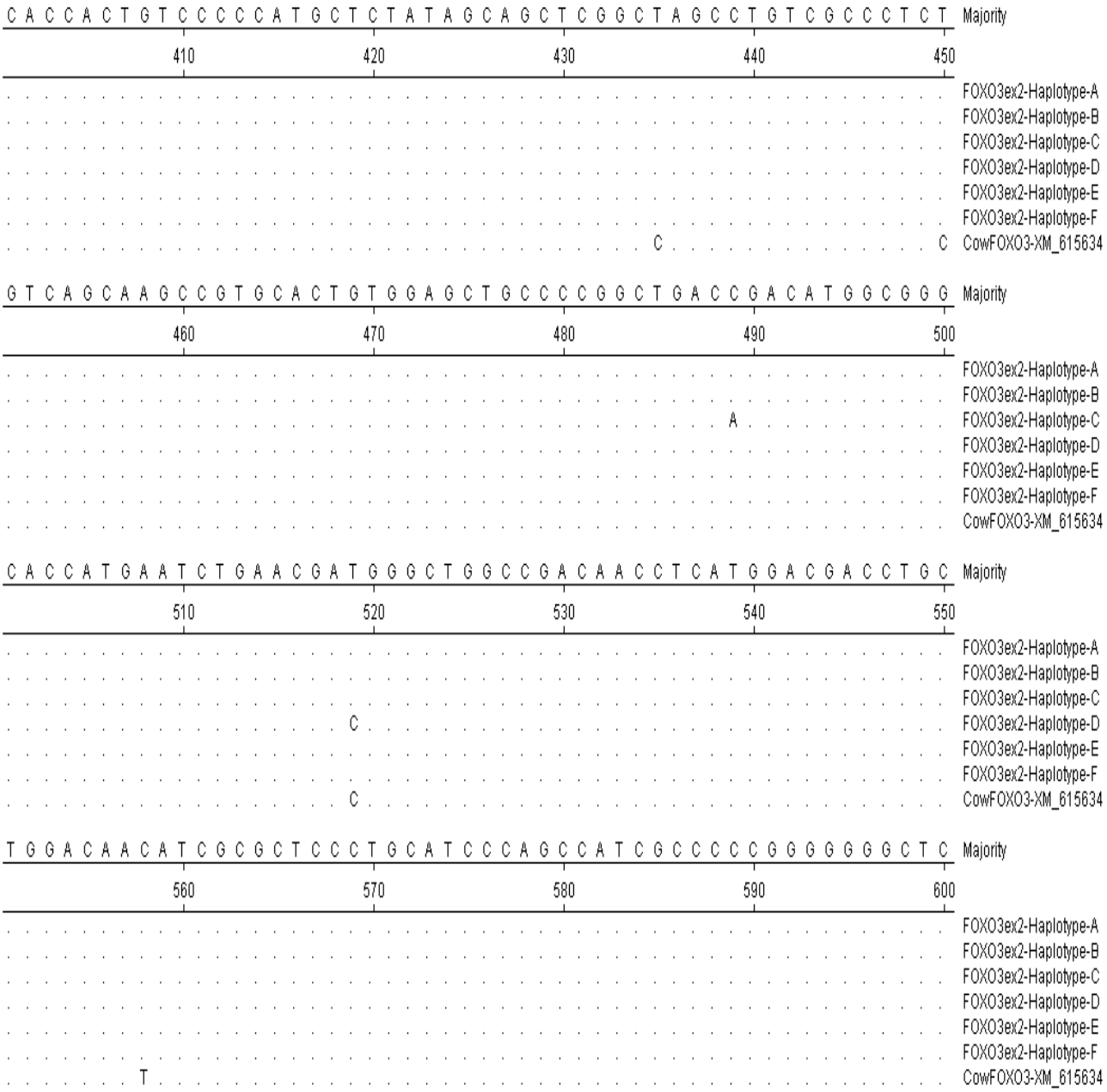
IGF1R exon 3 *A* to *C*: Accession number EF669473 to EF669475
Bovine IGF1R: Accession number NM_001244612.1

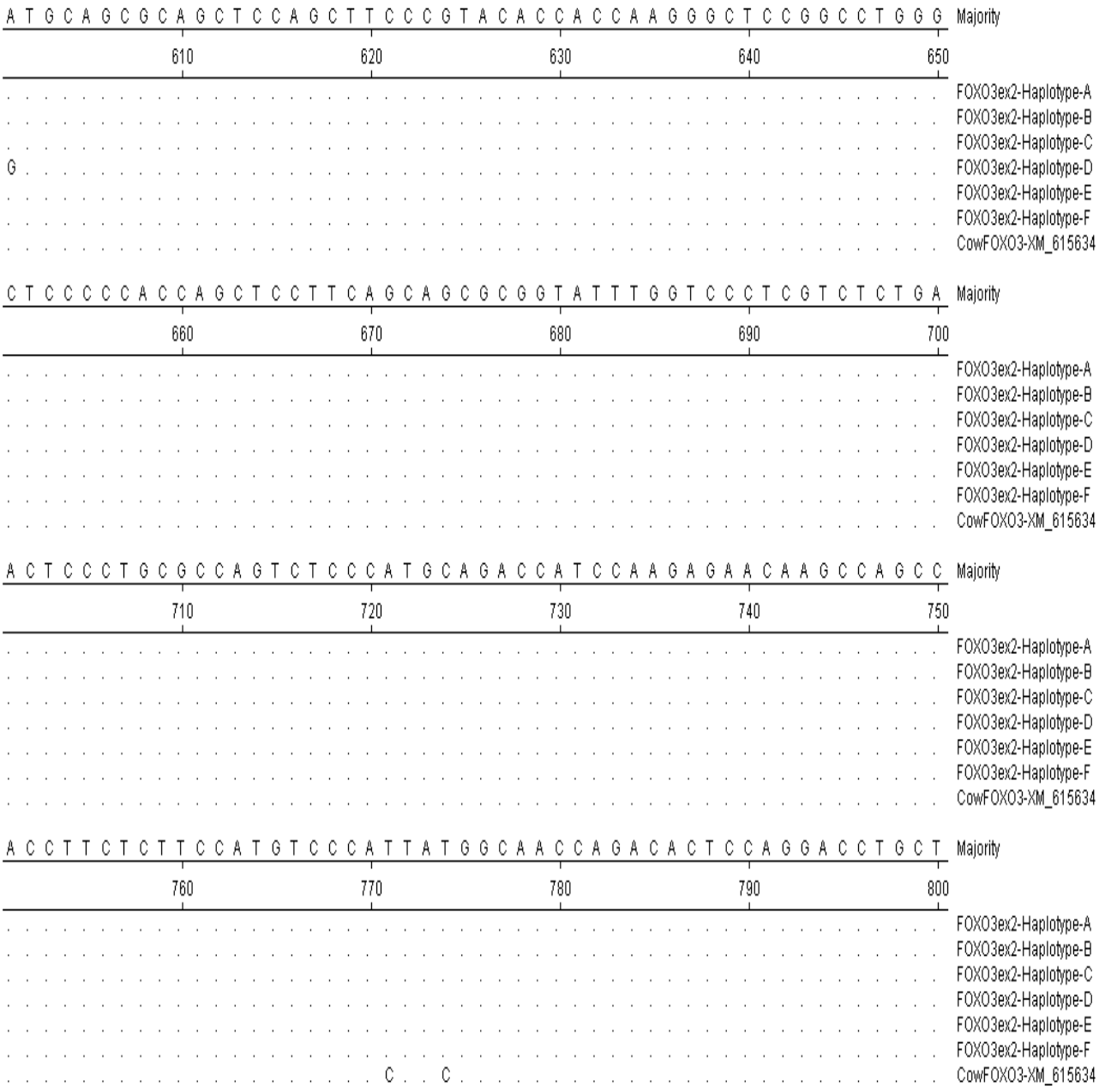
Appendix E: Exon 2 of ovine *FOXO3* (alignment of haplotypes *A* through *G* with exon 2 from cattle)

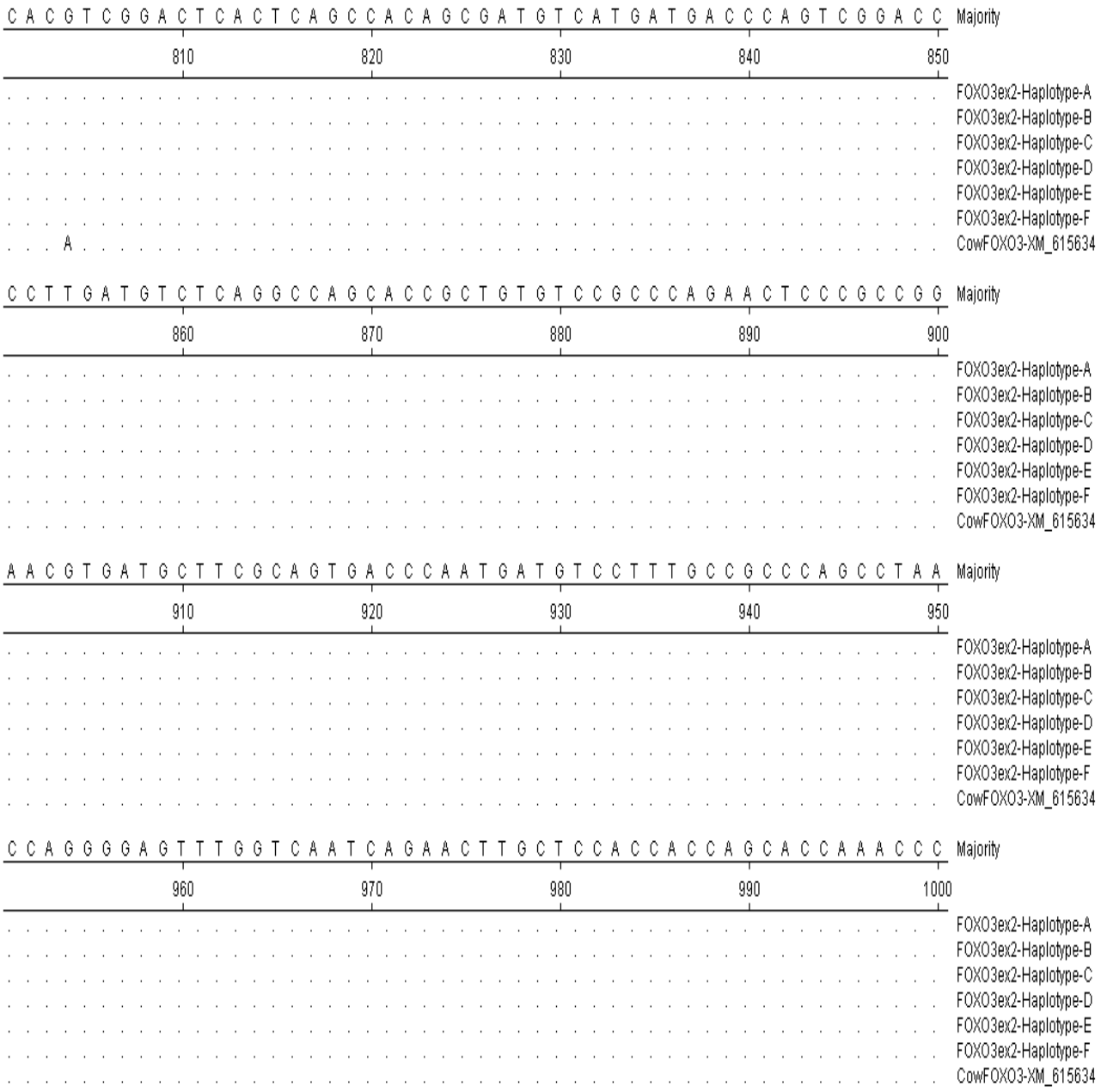


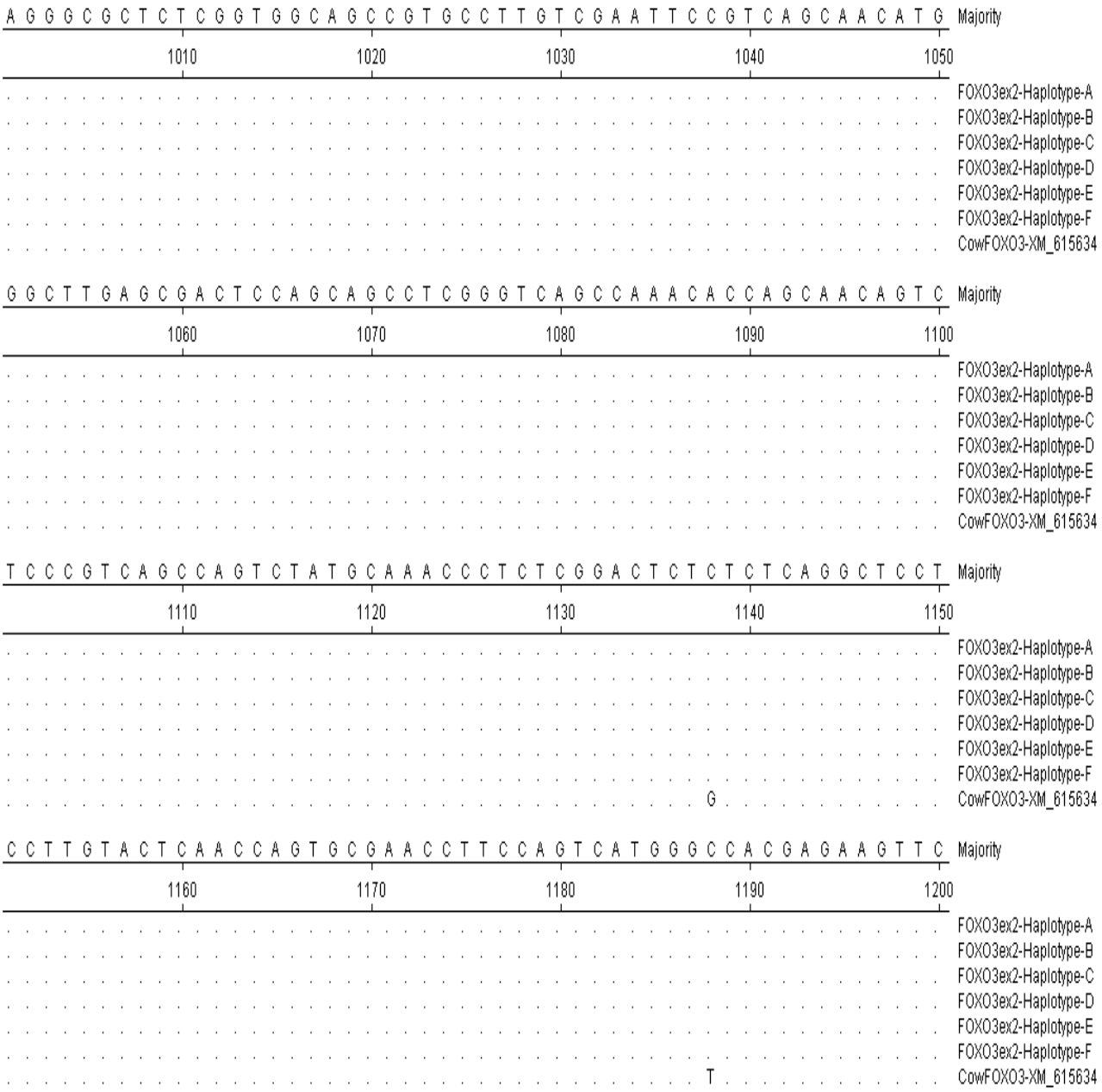
Haplotype *A* to *G*: Accession number GQ995521 to GQ995527
Bovine FOXO3: Accession number XM_615634.1

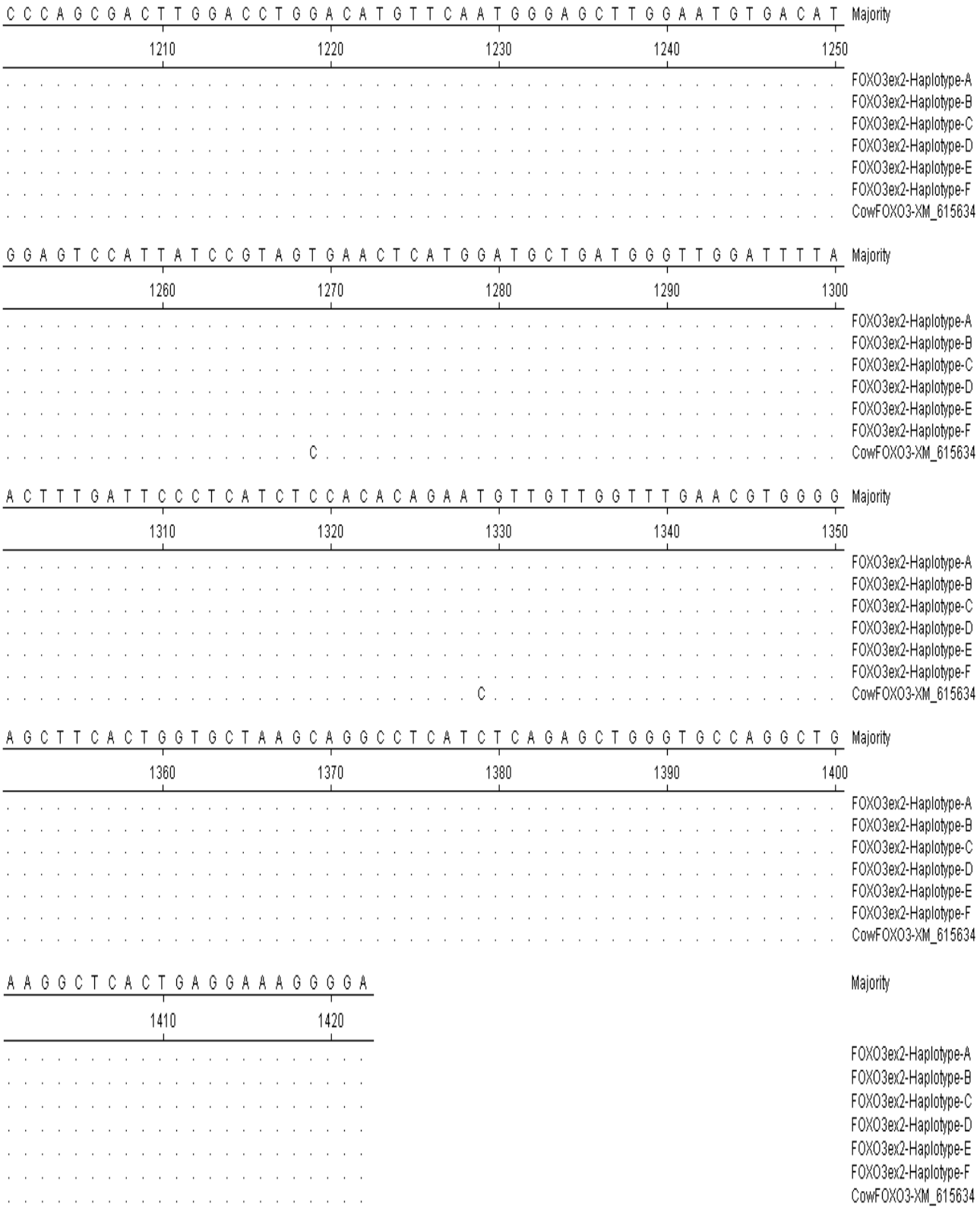

G C A G A C C G C C C C C G A A T C A G C A G A C G A C A G T C C C T C C C A G C T C T C C A A G T Majority
210 220 230 240 250
FOXO3ex2-Haplotype-A
FOXO3ex2-Haplotype-B
FOXO3ex2-Haplotype-C
FOXO3ex2-Haplotype-D
FOXO3ex2-Haplotype-E
FOXO3ex2-Haplotype-F
CowFOXO3-XM_615634
G G C C C G G C A G C C C C A C A T C C C G C A G C A G C G A T G A G C T G G A C G C G T G G A C C Majority
260 270 280 290 300
FOXO3ex2-Haplotype-A
FOXO3ex2-Haplotype-B
FOXO3ex2-Haplotype-C
FOXO3ex2-Haplotype-D
FOXO3ex2-Haplotype-E
FOXO3ex2-Haplotype-F
CowFOXO3-XM_615634
G A C T T C C G C T C A C G C A C C A A T T C C A A C G C C A G C A C A G T C A G C G G C C G C C T Majority
310 320 330 340 350
FOXO3ex2-Haplotype-A
FOXO3ex2-Haplotype-B
FOXO3ex2-Haplotype-C
FOXO3ex2-Haplotype-D
FOXO3ex2-Haplotype-E
FOXO3ex2-Haplotype-F
CowFOXO3-XM_615634
G T C C C C C A T T C T G G C G A G C A C A G A G C T G G A C G A T G T C C A G G A C G A T G A C G Majority
360 370 380 390 400
FOXO3ex2-Haplotype-A
FOXO3ex2-Haplotype-B
FOXO3ex2-Haplotype-C
FOXO3ex2-Haplotype-D
FOXO3ex2-Haplotype-E
FOXO3ex2-Haplotype-F
CowFOXO3-XM_615634


C A C C A C T G T C C C C C A T G C T C T A T A G C A G C T C G G C T A G C C T G T C G C C C T C T Majority
410 420 430 440 450
FOXO3ex2-Haplotype-A
FOXO3ex2-Haplotype-B
FOXO3ex2-Haplotype-C
FOXO3ex2-Haplotype-D
FOXO3ex2-Haplotype-E
FOXO3ex2-Haplotype-F
C C CowFOXO3-XM_615634
G T C A G C A A G C C G T G C A C T G T G G A G C T G C C C C G G C T G A C C G A C A T G G C G G G Majority
460 470 480 490 500
FOXO3ex2-Haplotype-A
FOXO3ex2-Haplotype-B
A FOXO3ex2-Haplotype-C
FOXO3ex2-Haplotype-D
FOXO3ex2-Haplotype-E
FOXO3ex2-Haplotype-F
CowFOXO3-XM_615634
C A C C A T G A A T C T G A A C G A T G G G C T G G C C G A C A A C C T C A T G G A C G A C C T G C Majority
510 520 530 540 550
FOXO3ex2-Haplotype-A
FOXO3ex2-Haplotype-B
FOXO3ex2-Haplotype-C
C FOXO3ex2-Haplotype-D
FOXO3ex2-Haplotype-E
FOXO3ex2-Haplotype-F
C CowFOXO3-XM_615634
T G G A C A A C A T C G C G C T C C C T G C A T C C C A G C C A T C G C C C C C G G G G G G G C T C Majority
560 570 580 590 600
FOXO3ex2-Haplotype-A
FOXO3ex2-Haplotype-B
FOXO3ex2-Haplotype-C
FOXO3ex2-Haplotype-D
FOXO3ex2-Haplotype-E
FOXO3ex2-Haplotype-F
T CowFOXO3-XM_615634


ATGCAGCGCAGCTCCAGCTTCCCGTACACCACCAAGGGCTCCGGCCTGGG Majority
610 620 630 640 650
FOXO3ex2-Haplotype-A
FOXO3ex2-Haplotype-B
FOXO3ex2-Haplotype-C
G FOXO3ex2-Haplotype-D
FOXO3ex2-Haplotype-E
FOXO3ex2-Haplotype-F
CowFOXO3-XM_615634
CTCCCCCACCAGCTCCTTCAGCAGCGCGGTATTTGGTCCCTCGTCTCTGA Majority
660 670 680 690 700
FOXO3ex2-Haplotype-A
FOXO3ex2-Haplotype-B
FOXO3ex2-Haplotype-C
FOXO3ex2-Haplotype-D
FOXO3ex2-Haplotype-E
FOXO3ex2-Haplotype-F
CowFOXO3-XM_615634
ACTCCCTGCGCCAGTCTCCCATGCAGACCATCCAAGAGAACAAGCCAGCC Majority
710 720 730 740 750
FOXO3ex2-Haplotype-A
FOXO3ex2-Haplotype-B
FOXO3ex2-Haplotype-C
FOXO3ex2-Haplotype-D
FOXO3ex2-Haplotype-E
FOXO3ex2-Haplotype-F
CowFOXO3-XM_615634
ACCTTCTCTTCCATGTCCCATTATGGCAACCAGACACTCCAGGACCTGCT Majority
760 770 780 790 800
FOXO3ex2-Haplotype-A
FOXO3ex2-Haplotype-B
FOXO3ex2-Haplotype-C
FOXO3ex2-Haplotype-D
FOXO3ex2-Haplotype-E
FOXO3ex2-Haplotype-F
C C CowFOXO3-XM_615634


C A C G T C G G A C T C A C T C A G C C A C A G C G A T G T C A T G A T G A C C C A G T C G G A C C Majority
810 820 830 840 850
FOXO3ex2-Haplotype-A
FOXO3ex2-Haplotype-B
FOXO3ex2-Haplotype-C
FOXO3ex2-Haplotype-D
FOXO3ex2-Haplotype-E
FOXO3ex2-Haplotype-F
A CowFOXO3-XM_615634
C C T T G A T G T C T C A G G C C A G C A C C G C T G T G T C C G C C C A G A A C T C C C G C C G G Majority
860 870 880 890 900
FOXO3ex2-Haplotype-A
FOXO3ex2-Haplotype-B
FOXO3ex2-Haplotype-C
FOXO3ex2-Haplotype-D
FOXO3ex2-Haplotype-E
FOXO3ex2-Haplotype-F
CowFOXO3-XM_615634
A A C G T G A T G C T T C G C A G T G A C C C A A T G A T G T C C T T T G C C G C C C A G C C T A A Majority
910 920 930 940 950
FOXO3ex2-Haplotype-A
FOXO3ex2-Haplotype-B
FOXO3ex2-Haplotype-C
FOXO3ex2-Haplotype-D
FOXO3ex2-Haplotype-E
FOXO3ex2-Haplotype-F
CowFOXO3-XM_615634
C C A G G G A G T T T G G T C A A T C A G A A C T T G C T C C A C C A C C A G C A C C A A A C C C Majority
960 970 980 990 1000
FOXO3ex2-Haplotype-A
FOXO3ex2-Haplotype-B
FOXO3ex2-Haplotype-C
FOXO3ex2-Haplotype-D
FOXO3ex2-Haplotype-E
FOXO3ex2-Haplotype-F
CowFOXO3-XM_615634


A G G G C G C T C T C G G T G G C A G C C G T G C C T T G T C G A A T T C C G T C A G C A A C A T G Majority
1010 1020 1030 1040 1050
FOXO3ex2-Haplotype-A
FOXO3ex2-Haplotype-B
FOXO3ex2-Haplotype-C
FOXO3ex2-Haplotype-D
FOXO3ex2-Haplotype-E
FOXO3ex2-Haplotype-F
CowFOXO3-XM_615634
G G C T T G A G C G A C T C C A G C A G C C T C G G G T C A G C C A A A C A C C A G C A A C A G T C Majority
1060 1070 1080 1090 1100
FOXO3ex2-Haplotype-A
FOXO3ex2-Haplotype-B
FOXO3ex2-Haplotype-C
FOXO3ex2-Haplotype-D
FOXO3ex2-Haplotype-E
FOXO3ex2-Haplotype-F
CowFOXO3-XM_615634
T C C C G T C A G C C A G T C T A T G C A A A C C C T C T C G G A C T C T C T C T C A G G C T C C T Majority
1110 1120 1130 1140 1150
FOXO3ex2-Haplotype-A
FOXO3ex2-Haplotype-B
FOXO3ex2-Haplotype-C
FOXO3ex2-Haplotype-D
FOXO3ex2-Haplotype-E
FOXO3ex2-Haplotype-F
G
CowFOXO3-XM_615634
C C T T G T A C T C A A C C A G T G C G A A C C T T C C A G T C A T G G G C C A C G A G A A G T T C Majority
1160 1170 1180 1190 1200
FOXO3ex2-Haplotype-A
FOXO3ex2-Haplotype-B
FOXO3ex2-Haplotype-C
FOXO3ex2-Haplotype-D
FOXO3ex2-Haplotype-E
FOXO3ex2-Haplotype-F
T
CowFOXO3-XM_615634


C C C A G C G A C T T G G A C C T G G A C A T G T T C A A T G G G A G C T T G G A A T G T G A C A T
Majority
1210
1220
1230
1240
1250
FOXO3ex2-Haplotype-A
FOXO3ex2-Haplotype-B
FOXO3ex2-Haplotype-C
FOXO3ex2-Haplotype-D
FOXO3ex2-Haplotype-E
FOXO3ex2-Haplotype-F
CowFOXO3-XM_615634
G G A G T C C A T T A T C C G T A G T G A A C T C A T G G A T G C T G A T G G G T T G G A T T T T A
Majority
1260
1270
1280
1290
1300
FOXO3ex2-Haplotype-A
FOXO3ex2-Haplotype-B
FOXO3ex2-Haplotype-C
FOXO3ex2-Haplotype-D
FOXO3ex2-Haplotype-E
FOXO3ex2-Haplotype-F
C
CowFOXO3-XM_615634
A C T T T G A T T C C C T C A T C T C C A C A C A G A A T G T T G T T G G T T T G A A C G T G G G G
Majority
1310
1320
1330
1340
1350
FOXO3ex2-Haplotype-A
FOXO3ex2-Haplotype-B
FOXO3ex2-Haplotype-C
FOXO3ex2-Haplotype-D
FOXO3ex2-Haplotype-E
FOXO3ex2-Haplotype-F
C
CowFOXO3-XM_615634
A G C T T C A C T G G T G C T A A G C A G G C C T C A T C T C A G A G C T G G G T G C C A G G C T G
Majority
1360
1370
1380
1390
1400
FOXO3ex2-Haplotype-A
FOXO3ex2-Haplotype-B
FOXO3ex2-Haplotype-C
FOXO3ex2-Haplotype-D
FOXO3ex2-Haplotype-E
FOXO3ex2-Haplotype-F
CowFOXO3-XM_615634
A A G G C T C A C T G A G G A A A G G G G A
Majority
1410
1420
FOXO3ex2-Haplotype-A
FOXO3ex2-Haplotype-B
FOXO3ex2-Haplotype-C
FOXO3ex2-Haplotype-D
FOXO3ex2-Haplotype-E
FOXO3ex2-Haplotype-F
CowFOXO3-XM_615634

Appendix F: Alignment of full coding sequence of *FOXO3* in sheep, cattle, human and Xenopus sp.

```
FOXO3-ovine    ATGGCAGAGGCGCCGGCCTCCCCGGCCCCGATCTCTCCGCTGGAAGTGGA     50
FOXO3-bovine   ----------------------------------------c--------     50
FOXO3-human    ----------a-----t-----------c----------c--------     50
FOXO3-Xenopus --------a--a-t-.--------ct---cgc--ga-.....--c-----     44

FOXO3-ovine    GCTGGACCCGGAGTTCGAGCCCCAGAGCCGGCCGCGCTCCTGTACGTGGC    100
FOXO3-bovine   ---a------------------------------------------------    100
FOXO3-human    ----------------------------t-----a-------------    100
FOXO3-Xenopus ca-a--------c----g---g-----t--a--t--g--t--c-------     94

FOXO3-ovine    CCCTGCAGAGGCCGGAGCTCCAGGGGAGCCCGGCCAAGCCCTCTGGGGAG    150
FOXO3-bovine   ------------------------------------------------    150
FOXO3-human    -------a--------------a-c------t-----------g------    150
FOXO3-Xenopus ----------a-ta--ctc---a--c-------g------gaa-a-t-gc    144

FOXO3-ovine    GCGGCTGCTGACTCCATGATCCCCGAGGAGGAGGACGATGAAGACGACGA    200
FOXO3-bovine   ------------------------------------------------    200
FOXO3-human    a----c--c-------------------------------------------    200
FOXO3-Xenopus --t-gg-aa-c-g-agac-cgt--tctat-atcccg--g--g--g--t--    194

FOXO3-ovine    GGACGGTGGCGGTAGGGCCGGCTCGGCCATGGCGATCGGTGGCGGCGG..    248
FOXO3-bovine   ----------------------------------------c--------..    248
FOXO3-human    ------c--g--ac-------------------------c--------cg    250
FOXO3-Xenopus t---ta--ag--agct--ta-ta-a----ct-t-.ct--g-a-a----..    241

FOXO3-ovine    .GGGCGGCCCGCTGGGCTCTGGGTTACTCCTGGAGGACTCGGCCCGGCTC    297
FOXO3-bovine   .------------------------g----------------------g    297
FOXO3-human    g-a-----a----------c---c-g-----t---------------g-g    300
FOXO3-Xenopus .....................---ac-.......---g-a--ttggt---g    264

FOXO3-ovine    TTGGCTCCTGGAGGGCAGGACCCCGGGTCCGGGCCAGCCCCCGCGGCGGG    347
FOXO3-bovine   c------------------------------------------------    347
FOXO3-human    c----a--c--------a-----------t---------a----------    350
FOXO3-Xenopus c--agcggc----aatc--ga-aact-g---t--tg--gt-ac-t-tt--    314

FOXO3-ovine    CGCGCTGAGCGGGGGAACGCAGACACCGCTGCAGCCTCAGCAGCCACTGC    397
FOXO3-bovine   ---------------g--------------------------------    397
FOXO3-human    --g------------t--a---g-g-t---------------a--g----    400
FOXO3-Xenopus g-g-g--...-aaacgct----gttt----cgg-gg-..-a--gag-a-g    359

FOXO3-ovine    CACCGCCGCAGCCGGGGACGGCTGGGGGCTCCGGGCAGCCGAGGAAATGC    447
FOXO3-bovine   ------------------------------t-----------------    447
FOXO3-human    -----------------g--------------------------------t    450
FOXO3-Xenopus ggg-----tgag---t-....-a--a--acagca-----a---------t    405

FOXO3-ovine    TCGTCGCGGAGGAACGCCTGGGGGAACCTGTCCTACGCCGACCTGATCAC    497
FOXO3-bovine   ----------------------------------------t--------    497
FOXO3-human    ---------c-------------a--------------g-----------    500
FOXO3-Xenopus --a--c--c--a--t--------c---a----------t-----t-----    455

FOXO3-ovine    TCGCGCGATCGAGAGCTCCCCAGACAAACGGCTCACTCTGTCCCAGATCT    547
FOXO3-bovine   ------------------t------------------------------    547
FOXO3-human    c-----c--------------g----------------------------    550
FOXO3-Xenopus -a-g--c---------a---ag--t-----------c-------------    505
```

```
FOXO3-ovine   ATGAGTGGATGGTGCGCTGCGTGCCCTACTTCAAGGATAAGGGCGACAGC    597
FOXO3-bovine  ------------------------------------------------    597
FOXO3-human   -c--------------t---------------------------------    600
FOXO3-Xenopus ----t--------c----c-----------------------a------    555

FOXO3-ovine   AACAGCTCTGCCGGCTGGAAGAACTCTATCCGGCACAACCTGTCACTGCA    647
FOXO3-bovine  ------------------------------------------------    647
FOXO3-human   -------------------------c----------------------    650
FOXO3-Xenopus -----------a-----------t--a--------t--t--------a--    605

FOXO3-ovine   CAGCCGATTCATGCGGGTCCAGAATGAGGGGACCGGCAAGAGCTCATGGT    697
FOXO3-bovine  ------g----------------------a-------------g----    697
FOXO3-human   t--t-------------------------a--t-----------t----    700
FOXO3-Xenopus t---a-g--t--ta-a--t-----------ct-t--a--a-----t----    655

FOXO3-ovine   GGATCATCAACCCGGATGGCGGGAAGAGTGGCAAGGCACCCCGGCGGCGG    747
FOXO3-bovine  -------------t--c-------------------g------------    747
FOXO3-human   -------------t-----g--------c--a--a--c------------    750
FOXO3-Xenopus ----g--------a--g--t--t--ag----a--------aa-aa-a--t    705

FOXO3-ovine   GCCGTTTCCATGGACAACAGCAACAAGTACACCAAGAGCCGCGGCCGTGC    797
FOXO3-bovine  -----c------------------------------------------    797
FOXO3-human   --t--c-----------t-----------t-----------t-----c--    800
FOXO3-Xenopus --t-----a--------t--t-----------------t-----ga-a--    755

FOXO3-ovine   CGCCAAGAAGAAGGCAGCCCTGCAGACCGCCCCCGAATCAGCAGACGACA    847
FOXO3-bovine  a-----------------------------------g--------t----    847
FOXO3-human   a--------------------------a--------------t-------    850
FOXO3-Xenopus a--a-----a--a---t--t-----g-at-tt-t--tg-ta----t--t-    805

FOXO3-ovine   GTCCCTCCCAGCTCTCCAAGTGGCCCGGCAGCCCCACATCCCGCAGCAGC    897
FOXO3-bovine  ------------------------------------------------    897
FOXO3-human   -------------------------t-----------g--a--------t    900
FOXO3-Xenopus -c--t--a-----g--a--------a--t--t-----c--a--t--t--t    855

FOXO3-ovine   GATGAGCTGGACGCGTGGACCGACTTCCGCTCACGCACCAATTCCAACGC    947
FOXO3-bovine  --------------------------------g-----------------    947
FOXO3-human   -----------t--------g--------t--------------t-----    950
FOXO3-Xenopus ---a-a--t--ta-c-----a-----t--a--t--t--a--c--t--t--    905

FOXO3-ovine   CAGCACAGTCAGCGGCCGCCTGTCCCCCATTCTGGCGAGCACAGAGCTGG    997
FOXO3-bovine  --------------------------------c-----------g-------    997
FOXO3-human   ------------t-----------g-----ca----a---------t---   1000
FOXO3-Xenopus ---t--ta-a--t--t--gt----t--a----ca--t-c---t--a--t-    955

FOXO3-ovine   ACGATGTCCAGGACGATGACGCACCACTGTCCCCCATGCTCTATAGCAGC   1047
FOXO3-bovine  ----c--------------t-------------------------------   1047
FOXO3-human   -t--a--------------t--g--t--c--g-----------c------   1050
FOXO3-Xenopus -t-----t--a--t-----tt-t--c--------t---t-g----a----   1005

FOXO3-ovine   TCGGCTAGCCTGTCGCCCTCTGTCAGCAAGCCGTGCACTGTGGAGCTGCC   1097
FOXO3-bovine  -----c--------------c-------------------------------   1097
FOXO3-human   --a--c--------a--t--a--a--------------g-----a-----   1100
FOXO3-Xenopus c-a-g---tt----c--a--ca-a--t--a--a-----a------a----   1055

FOXO3-ovine   CCGGCTGACCGACATGGCGGGCACCATGAATCTGAACGATGGGCTGGCCG   1147
FOXO3-bovine  ----------------------------------------c----------   1147
FOXO3-human   a--------t--t-----a-----------------t---------a-t-   1150
FOXO3-Xenopus aa-aa-a--t--t-----t-aa--------ct-a--t--c--at--c-g-   1105
```

```
FOXO3-ovine   ACAACCTCATGGACGACCTGCTGGACAACATCGCGCTCCCTGCATCCCAG   1197
FOXO3-bovine  ----------------------------t--------------------   1197
FOXO3-human   -a------------------------t------a-------gc--------   1200
FOXO3-Xenopus -g-----------t---t-------tg----tt-t---a--t-t--a---   1155

FOXO3-ovine   CCATCGCCCCCGGGGGGGCTCATGCAGCGCAGCTCCAGCTTCCCGTACAC   1247
FOXO3-bovine  ------------------------------------------------   1247
FOXO3-human   ---------a-t-----a-----------g-----t-----------t--   1250
FOXO3-Xenopus -a---at-t--c--t-tc---------a-a--t--------ta-a--tgg   1205

FOXO3-ovine   CACCAAGGGCTCCGGCCTGGGCTCCCCCACCAGCTCCTTCAGCAGCGCGG   1297
FOXO3-bovine  ------------------------------------------------   1297
FOXO3-human   ------------g--------------a-----------t-a----a---   1300
FOXO3-Xenopus ---t--------a---a-t-----t--at-g-ataat--t-a--a-a-t-   1255

FOXO3-ovine   TATTTGGTCCCTCGTCTCTGAACTCCCTGCGCCAGTCTCCCATGCAGACC   1347
FOXO3-bovine  ------------------------------------------------   1347
FOXO3-human   -g--c--a--t--a--------------a--------------------   1350
FOXO3-Xenopus gtagcttcaa--ttc-ct---ca--t--a-----------------a--g   1305

FOXO3-ovine   ATCCAAGAGAACAAGCCAGCCACCTTCTCTTCCATGTCCCATTATGGCAA   1397
FOXO3-bovine  ----------------------------------------c--c-----   1397
FOXO3-human   --------------------t-----------------a--c-----t--   1400
FOXO3-Xenopus --------------a-a---t--a--t---------aat------a-t--   1355

FOXO3-ovine   CCAGACACTCCAGGACCTGCTCACGTCGGACTCACTCAGCCACAGCGATG   1447
FOXO3-bovine  ------------------------a------------------------   1447
FOXO3-human   ------------------------t----------t-------------   1450
FOXO3-Xenopus ----t-tt-g--a------t-g-ata-a---a-c----------------   1405

FOXO3-ovine   TCATGATGACCCAGTCGGACCCCTTGATGTCTCAGGCCAGCACCGCTGTG   1497
FOXO3-bovine  ------------------------------------------------   1497
FOXO3-human   ----------a-------------------------------------   1500
FOXO3-Xenopus -gt-a-----------t--t--gc-c-----a--a-----t--t--a--a   1455

FOXO3-ovine   TCCGCCCAGAACTCCCGCCGGAACGTGATGCTTCGCAGTGACCCAATGAT   1547
FOXO3-bovine  ------------------------------------------------   1547
FOXO3-human   --t--------t-------------------------a---t--g-----   1550
FOXO3-Xenopus a-t--t-----tag-a-aa-a--ta-c--t--ga-a-a---t--t-----   1505

FOXO3-ovine   GTCCTTTGCCGCCCAGCCTAACCAGGGGAG...TTTGGTCAATCAGAACT   1594
FOXO3-bovine  ------------------------------...-----------------   1594
FOXO3-human   ---------t----------------a--...-----------------   1597
FOXO3-Xenopus ---t-----a-----a--------a--ag-aaac-----t-----a----   1555

FOXO3-ovine   TGCTCCACCACCAGCACCAAACCCAGGGCGCTCTCGGTGGCAGCCGTGCC   1644
FOXO3-bovine  ------------------------------------------------   1644
FOXO3-human   ----------------------------------t---------------   1647
FOXO3-Xenopus c---g-----g-----gtctctta-ctcttt--aa---------------   1605

FOXO3-ovine   TTGTCGAATTCCGTCAGCAACATGGGCTTGAGCGACTCCAGCAGCCTCGG   1694
FOXO3-bovine  ------------------------------------------------   1694
FOXO3-human   -----------t--------------------t--g-----------t--   1697
FOXO3-Xenopus -----a--caatt-a-------ct--t--a-at---ag-----t-t-g-a   1655

FOXO3-ovine   GTCAGCCAAACACCAGCAACAGTCTCCCGTCAGCCAGTCTATGCAAACCC   1744
FOXO3-bovine  ------------------------------------------------   1744
FOXO3-human   ------------------g--------t---------------------   1747
```

```
FOXO3-Xenopus a---a-------------g-----ct-a--g--t--t------------a   1705

FOXO3-ovine   TCTCGGACTCTCTCTCAGGCTCCTCCTTGTACTCAACCAGTGCGAACCTT   1794
FOXO3-bovine  -----------g-------------------------------------   1794
FOXO3-human   ----------------------------------t-----a-----g   1797
FOXO3-Xenopus ----c---a-g--------a--...---------ct-ag-c-t--c----   1752

FOXO3-ovine   CCAGTCATGGGCCACGAGAAGTTCCCCAGCGACTTGGACCTGGACATGTT   1844
FOXO3-bovine  -----------t-------------------------------------   1844
FOXO3-human   --c-----------t-------------------------------------   1847
FOXO3-Xenopus ---acac----a--t--a-----t----ct--t-----------t--t--   1802

FOXO3-ovine   CAATGGGAGCTTGGAATGTGACATGGAGTCCATTATCCGTAGTGAACTCA   1894
FOXO3-bovine  ----------------------------------------c-------   1894
FOXO3-human   -------------------------------------------------   1897
FOXO3-Xenopus ---------t----------t------a-g--a--t--c-ac--t----   1852

FOXO3-ovine   TGGATGCTGATGGGTTGGATTTTAACTTTGATTCCCTCATCTCCACACAG   1944
FOXO3-bovine  -------------------------------------------------   1944
FOXO3-human   -------------------------------------------------   1947
FOXO3-Xenopus -------a-----------------t-----ca----------ag-t---   1902

FOXO3-ovine   AATGTTGTTGGTTTGAACGTGGGGAGCTTCACTGGTGCTAAGCAGGCCTC   1994
FOXO3-bovine  --c-----------------------------------------------   1994
FOXO3-human   -------------------------a-----------------------   1997
FOXO3-Xenopus --...---ca--c-atct--a--c--t-----------------a-a--   1949

FOXO3-ovine   ATCTCAGAGCTGGGTGCCAGGCTGA                           2019
FOXO3-bovine  -------------------------                           2019
FOXO3-human   -------------------------                           2022
FOXO3-Xenopus ---a--------------------                            1974
```

Ovine FOXO3: Accession number JQ894783
Bovine FOXO3: Accession number XM_615634
Human FOXO3: Accession number NM_201559
Xenopus FOXO3: Accession number NM_001092949

Appendix G: Statistical analyses for association of longevity and variation in *IGF1R*, *FOXO3*, *CAST* and *TLR4*-breed correction model

Univariate Analysis of Variance-IGF1R A

Dependent Variable:age

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	5191.638	1	5191.638	88.567	.000
	Error	398.633	6.801	58.618[a]		
Breed	Hypothesis	2448.573	5	489.715	52.583	.000
	Error	15934.961	1711	9.313[b]		
IGF1RA	Hypothesis	1.760	1	1.760	.189	.664
	Error	15934.961	1711	9.313[b]		

a. .103 MS(Breed) + .897 MS(Error)

b. MS(Error)

Estimated Marginal Means-IGF1R A

Dependent Variable:age

IGF1RA	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	5.409	.458	4.510	6.308
1	5.611	.082	5.451	5.771

Univariate Analysis of Variance-IGF1R B

Dependent Variable:age

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	19351.782	1	19351.782	101.353	.000
	Error	1015.402	5.318	190.935[a]		
Breed	Hypothesis	2452.384	5	490.477	52.665	.000
	Error	15934.841	1711	9.313[b]		
IGF1RB	Hypothesis	1.880	1	1.880	.202	.653
	Error	15934.841	1711	9.313[b]		

a. .377 MS(Breed) + .623 MS(Error)

b. MS(Error)

Estimated Marginal Means-IGF1R B

Dependent Variable:age

IGF1RB	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	5.618	.085	5.450	5.786
1	5.510	.227	5.065	5.955

Univariate Analysis of Variance-IGF1R C

Dependent Variable:age

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	26202.910	1	26202.910	119.060	.000
	Error	1151.182	5.231	220.081[a]		
Breed	Hypothesis	2284.738	5	456.948	49.257	.000
	Error	15872.661	1711	9.277[b]		
IGF1RC	Hypothesis	64.060	1	64.060	6.905	.009
	Error	15872.661	1711	9.277[b]		

a. .471 MS(Breed) + .529 MS(Error)

b. MS(Error)

Estimated Marginal Means-IGF1R C

Dependent Variable:age

IGF1RC	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	5.519	.087	5.348	5.689
1	6.079	.197	5.692	6.466

Univariate Analysis of Variance-FOXO3 A

Dependent Variable:age

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	23355.518	1	23355.518	107.885	.000
	Error	1354.616	6.257	216.485[a]		
FOXO3A	Hypothesis	4.265	1	4.265	.478	.490
	Error	15387.303	1724	8.925[b]		
Breed	Hypothesis	2564.154	6	427.359	47.881	.000
	Error	15387.303	1724	8.925[b]		

a. .496 MS(Breed) + .504 MS(Error)

b. MS(Error)

Estimated Marginal Means-FOXO3 A

Dependent Variable:age

FOXO3A	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	5.508	.164	5.186	5.830
1	5.385	.107	5.175	5.595

Univariate Analysis of Variance-FOXO3 B

Dependent Variable:age

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	27254.011	1	27254.011	111.729	.000
	Error	1509.542	6.188	243.930[a]		
Breed	Hypothesis	2481.212	6	413.535	46.320	.000
	Error	15391.552	1724	8.928[b]		
FOXO3B	Hypothesis	.017	1	.017	.002	.966
	Error	15391.552	1724	8.928[b]		

a. .581 MS(Breed) + .419 MS(Error)

b. MS(Error)

Estimated Marginal Means-FOXO3 B

Dependent Variable:age

FOXO3B	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	5.413	.118	5.182	5.645
1	5.420	.135	5.156	5.684

Univariate Analysis of Variance-FOXO3 C

Dependent Variable:age

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	2796.674	1	2796.674	88.241	.000
	Error	353.129	11.142	31.694[a]		
Breed	Hypothesis	2569.885	6	428.314	47.986	.000
	Error	15388.145	1724	8.926[b]		
FOXO3C	Hypothesis	3.424	1	3.424	.384	.536
	Error	15388.145	1724	8.926[b]		

a. .054 MS(Breed) + .946 MS(Error)

b. MS(Error)

Estimated Marginal Means-FOXO3 C

Dependent Variable:age

FOXO3C	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	5.411	.097	5.220	5.602
1	5.794	.618	4.582	7.006

Univariate Analysis of Variance-FOXO3 D

Dependent Variable:age

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	21126.783	1	21126.783	103.324	.000
	Error	1283.922	6.279	204.470[a]		
Breed	Hypothesis	2484.754	6	414.126	46.591	.000
	Error	15323.668	1724	8.888[b]		
FOXO3D	Hypothesis	67.900	1	67.900	7.639	.006
	Error	15323.668	1724	8.888[b]		

a. .483 MS(Breed) + .517 MS(Error)

b. MS(Error)

Estimated Marginal Means-FOXO3 D

Dependent Variable:age

FOXO3D	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	5.530	.105	5.323	5.736
1	5.035	.169	4.704	5.366

Univariate Analysis of Variance-FOXO3 E

Dependent Variable:age

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	6503.790	1	6503.790	93.911	.000
	Error	525.168	7.583	69.255[a]		
Breed	Hypothesis	2594.855	6	432.476	48.459	.000
	Error	15386.014	1724	8.925[b]		
FOXO3E	Hypothesis	5.554	1	5.554	.622	.430
	Error	15386.014	1724	8.925[b]		

a. .142 MS(Breed) + .858 MS(Error)

b. MS(Error)

Estimated Marginal Means-FOXO3 E

Dependent Variable:age

FOXO3E	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	5.429	.098	5.236	5.622
1	5.135	.369	4.412	5.859

Univariate Analysis of Variance-FOXO3 F

Dependent Variable:age

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	1885.930	1	1885.930	83.382	.000
	Error	354.332	15.666	22.618[a]		
Breed	Hypothesis	2595.961	6	432.660	48.515	.000
	Error	15374.702	1724	8.918[b]		
FOXO3F	Hypothesis	16.866	1	16.866	1.891	.169
	Error	15374.702	1724	8.918[b]		

a. .032 MS(Breed) + .968 MS(Error)

b. MS(Error)

Estimated Marginal Means-FOXO3 F

Dependent Variable:age

FOXO3F	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	5.409	.097	5.218	5.600
1	6.519	.808	4.934	8.104

Univariate Analysis of Variance-FOXO3 G

Dependent Variable:age

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	2599.752	1	2599.752	91.468	.000
	Error	347.183	12.215	28.423[a]		
Breed	Hypothesis	2613.693	6	435.616	48.855	.000
	Error	15371.894	1724	8.916[b]		
FOXO3G	Hypothesis	19.674	1	19.674	2.207	.138
	Error	15371.894	1724	8.916[b]		

a. .046 MS(Breed) + .954 MS(Error)

b. MS(Error)

Estimated Marginal Means-FOXO3 G

Dependent Variable:age

FOXO3G	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	5.391	.099	5.197	5.584
1	6.428	.688	5.078	7.778

Univariate Analysis of Variance-CAST A

Dependent Variable:age

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	44084.931	1	44084.931	106.311	.000
	Error	2086.800	5.032	414.678[a]		
Breed	Hypothesis	2411.407	5	482.281	51.651	.000
	Error	16050.713	1719	9.337[b]		
CASTA	Hypothesis	3.587	1	3.587	.384	.535
	Error	16050.713	1719	9.337[b]		

a. .857 MS(Breed) + .143 MS(Error)

b. MS(Error)

Estimated Marginal Means-CAST A

Dependent Variable:age

CASTA	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	5.565	.125	5.320	5.810
1	5.665	.104	5.461	5.869

Univariate Analysis of Variance-CAST B

Dependent Variable:age

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	34531.214	1	34531.214	104.928	.000
	Error	1677.271	5.097	329.094[a]		
Breed	Hypothesis	2453.694	5	490.739	52.566	.000
	Error	16047.949	1719	9.336[b]		
CASTB	Hypothesis	6.351	1	6.351	.680	.410
	Error	16047.949	1719	9.336[b]		

a. .664 MS(Breed) + .336 MS(Error)

b. MS(Error)

Estimated Marginal Means-CAST B

Dependent Variable:age

CASTB	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	5.739	.161	5.423	6.055
1	5.586	.092	5.405	5.768

Univariate Analysis of Variance-CAST C

Dependent Variable:age

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	24319.075	1	24319.075	117.079	.000
	Error	1092.653	5.260	207.715[a]		
Breed	Hypothesis	2292.241	5	458.448	49.166	.000
	Error	16028.815	1719	9.324[b]		
CASTC	Hypothesis	25.485	1	25.485	2.733	.098
	Error	16028.815	1719	9.324[b]		

a. .442 MS(Breed) + .558 MS(Error)

b. MS(Error)

Estimated Marginal Means-CAST C

Dependent Variable:age

CASTC	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	5.573	.086	5.403	5.742
1	5.937	.206	5.534	6.340

Univariate Analysis of Variance-CAST D

Dependent Variable:age

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	1276.539	1	1276.539	62.533	.000
	Error	333.048	16.315	20.414[a]		
Breed	Hypothesis	2447.612	5	489.522	52.417	.000
	Error	16053.606	1719	9.339[b]		
CASTD	Hypothesis	.695	1	.695	.074	.785
	Error	16053.606	1719	9.339[b]		

a. .023 MS(Breed) + .977 MS(Error)

b. MS(Error)

Estimated Marginal Means-CAST D

Dependent Variable:age

CASTD	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	5.623	.081	5.465	5.781
1	5.890	.980	3.969	7.812

Univariate Analysis of Variance-TLR4 A

Dependent Variable:age

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	43622.046	1	43622.046	102.540	.000
	Error	2139.410	5.029	425.417[a]		
Breed	Hypothesis	2441.797	5	488.359	52.292	.000
	Error	16054.005	1719	9.339[b]		
TLR4A	Hypothesis	.296	1	.296	.032	.859
	Error	16054.005	1719	9.339[b]		

a. .869 MS(Breed) + .131 MS(Error)

b. MS(Error)

Estimated Marginal Means-TLR4 A

Dependent Variable:age

TLR4A	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	5.606	.129	5.352	5.860
1	5.636	.105	5.431	5.841

Univariate Analysis of Variance-TLR4 B

Dependent Variable:age

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	44535.263	1	44535.263	103.756	.000
	Error	2157.675	5.027	429.230[a]		
Breed	Hypothesis	2440.535	5	488.107	52.295	.000
	Error	16044.809	1719	9.334[b]		
TLR4B	Hypothesis	9.491	1	9.491	1.017	.313
	Error	16044.809	1719	9.334[b]		

a. .877 MS(Breed) + .123 MS(Error)

b. MS(Error)

Estimated Marginal Means-TLR4 B

Dependent Variable:age

TLR4B	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	5.530	.123	5.288	5.772
1	5.695	.107	5.486	5.904

Univariate Analysis of Variance-TLR4 C

Dependent Variable:age

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	38206.976	1	38206.976	103.364	.000
	Error	1871.817	5.064	369.635[a]		
Breed	Hypothesis	2451.361	5	490.272	52.511	.000
	Error	16049.622	1719	9.337[b]		
TLR4C	Hypothesis	4.678	1	4.678	.501	.479
	Error	16049.622	1719	9.337[b]		

a. .749 MS(Breed) + .251 MS(Error)

b. MS(Error)

Estimated Marginal Means-TLR4 C

Dependent Variable:age

TLR4C	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	5.662	.096	5.473	5.851
1	5.537	.147	5.250	5.825

Univariate Analysis of Variance-TLR4 D

Dependent Variable:age

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	17190.807	1	17190.807	102.619	.000
	Error	902.776	5.389	167.521[a]		
Breed	Hypothesis	2374.243	5	474.849	50.889	.000
	Error	16040.186	1719	9.331[b]		
TLR4D	Hypothesis	14.114	1	14.114	1.513	.219
	Error	16040.186	1719	9.331[b]		

a. .340 MS(Breed) + .660 MS(Error)

b. MS(Error)

Estimated Marginal Means-TLR4 D

Dependent Variable:age

TLR4D	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	5.651	.083	5.487	5.814
1	5.352	.236	4.890	5.814

Appendix H: Statistical analyses for association of fecundity and variation in *IGF1R*, *FOXO3*, *CAST* and *TLR4*-breed correction model

Univariate Analysis of Variance-IGF1R A

Dependent Variable:fecundity

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	170.153	1	170.153	116.834	.000
	Error	6.929	4.758	1.456[a]		
Breed	Hypothesis	58.792	4	14.698	110.365	.000
	Error	86.166	647	.133[b]		
IGF1RA	Hypothesis	.000	1	.000	.002	.967
	Error	86.166	647	.133[b]		

a. .091 MS(Breed) + .909 MS(Error)

b. MS(Error)

Estimated Marginal Means-IGF1R A

Dependent Variable:fecundity

IGF1RA	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.725	.095	1.538	1.911
1	1.721	.016	1.690	1.751

Univariate Analysis of Variance-IGF1R B

Dependent Variable:fecundity

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	725.872	1	725.872	126.176	.000
	Error	23.678	4.116	5.753[a]		
Breed	Hypothesis	58.570	4	14.643	110.004	.000
	Error	86.122	647	.133[b]		
IGF1RB	Hypothesis	.044	1	.044	.331	.565
	Error	86.122	647	.133[b]		

a. .387 MS(Breed) + .613 MS(Error)

b. MS(Error)

Estimated Marginal Means-IGF1R B

Dependent Variable:fecundity

IGF1RB	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.717	.016	1.685	1.750
1	1.744	.044	1.658	1.830

Univariate Analysis of Variance-IGF1R C

Dependent Variable:fecundity

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	942.899	1	942.899	127.605	.000
	Error	30.096	4.073	7.389[a]		
Breed	Hypothesis	58.660	4	14.665	110.519	.000
	Error	85.852	647	.133[b]		
IGF1RC	Hypothesis	.313	1	.313	2.362	.125
	Error	85.852	647	.133[b]		

a. .499 MS(Breed) + .501 MS(Error)

b. MS(Error)

Estimated Marginal Means-IGF1R C

Dependent Variable:fecundity

IGF1RC	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.711	.017	1.678	1.744
1	1.773	.037	1.700	1.846

Univariate Analysis of Variance-FOXO3 A

Dependent Variable:fecundity

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	931.140	1	931.140	159.764	.000
	Error	29.830	5.118	5.828[a]		
Breed	Hypothesis	57.031	5	11.406	83.343	.000
	Error	85.401	624	.137[b]		
FOXO3A	Hypothesis	.131	1	.131	.960	.328
	Error	85.401	624	.137[b]		

a. .505 MS(Breed) + .495 MS(Error)

b. MS(Error)

Estimated Marginal Means-FOXO3 A

Dependent Variable:fecundity

FOXO3A	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.801	.034	1.733	1.869
1	1.837	.022	1.794	1.880

Univariate Analysis of Variance-FOXO3 B

Dependent Variable:fecundity

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	1061.110	1	1061.110	164.155	.000
	Error	32.927	5.094	6.464[a]		
Breed	Hypothesis	56.944	5	11.389	83.269	.000
	Error	85.345	624	.137[b]		
FOXO3B	Hypothesis	.187	1	.187	1.364	.243
	Error	85.345	624	.137[b]		

a. .562 MS(Breed) + .438 MS(Error)

b. MS(Error)

Estimated Marginal Means-FOXO3 B

Dependent Variable:fecundity

FOXO3B	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.842	.023	1.797	1.887
1	1.802	.030	1.743	1.861

Univariate Analysis of Variance-FOXO3 C

Dependent Variable:fecundity

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	101.395	1	101.395	137.595	.000
	Error	5.433	7.373	.737[a]		
Breed	Hypothesis	60.715	5	12.143	88.616	.000
	Error	85.506	624	.137[b]		
FOXO3C	Hypothesis	.026	1	.026	.188	.664
	Error	85.506	624	.137[b]		

a. .050 MS(Breed) + .950 MS(Error)

b. MS(Error)

Estimated Marginal Means-FOXO3 C

Dependent Variable:fecundity

FOXO3C	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.828	.020	1.789	1.867
1	1.886	.133	1.624	2.147

Univariate Analysis of Variance-FOXO3 D

Dependent Variable:fecundity

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	947.523	1	947.523	171.432	.000
	Error	28.321	5.124	5.527[a]		
Breed	Hypothesis	53.701	5	10.740	78.456	.000
	Error	85.422	624	.137[b]		
FOXO3D	Hypothesis	.110	1	.110	.800	.371
	Error	85.422	624	.137[b]		

a. .508 MS(Breed) + .492 MS(Error)

b. MS(Error)

Estimated Marginal Means-FOXO3 D

Dependent Variable:fecundity

FOXO3D	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.836	.022	1.794	1.879
1	1.804	.034	1.738	1.870

Univariate Analysis of Variance-FOXO3 E

Dependent Variable:fecundity

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	262.657	1	262.657	154.232	.000
	Error	9.843	5.780	1.703[a]		
Breed	Hypothesis	60.476	5	12.095	88.277	.000
	Error	85.497	624	.137[b]		
FOXO3E	Hypothesis	.035	1	.035	.252	.616
	Error	85.497	624	.137[b]		

a. .131 MS(Breed) + .869 MS(Error)

b. MS(Error)

Estimated Marginal Means-FOXO3 E

Dependent Variable:fecundity

FOXO3E	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.827	.020	1.788	1.867
1	1.867	.080	1.711	2.023

Univariate Analysis of Variance-FOXO3 F

Dependent Variable:fecundity

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	106.427	1	106.427	143.658	.000
	Error	5.446	7.351	.741[a]		
Breed	Hypothesis	60.723	5	12.145	88.756	.000
	Error	85.383	624	.137[b]		
FOXO3F	Hypothesis	.149	1	.149	1.088	.297
	Error	85.383	624	.137[b]		

a. .050 MS(Breed) + .950 MS(Error)

b. MS(Error)

Estimated Marginal Means-FOXO3 F

Dependent Variable:fecundity

FOXO3F	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.827	.020	1.788	1.866
1	1.966	.133	1.704	2.227

Univariate Analysis of Variance-FOXO3 G

Dependent Variable:fecundity

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	133.883	1	133.883	145.759	.000
	Error	6.199	6.749	.919[a]		
Breed	Hypothesis	61.015	5	12.203	89.164	.000
	Error	85.401	624	.137[b]		
FOXO3G	Hypothesis	.131	1	.131	.958	.328
	Error	85.401	624	.137[b]		

a. .065 MS(Breed) + .935 MS(Error)

b. MS(Error)

Estimated Marginal Means-FOXO3 G

Dependent Variable:fecundity

FOXO3G	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.824	.021	1.783	1.864
1	1.945	.120	1.708	2.181

Univariate Analysis of Variance-CAST A

Dependent Variable:fecundity

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	1513.570	1	1513.570	130.471	.000
	Error	46.591	4.016	11.601[a]		
Breed	Hypothesis	56.141	4	14.035	105.106	.000
	Error	87.064	652	.134[b]		
CASTA	Hypothesis	.000	1	.000	.002	.962
	Error	87.064	652	.134[b]		

a. .825 MS(Breed) + .175 MS(Error)

b. MS(Error)

Estimated Marginal Means-CAST A

Dependent Variable:fecundity

CASTA	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.720	.026	1.670	1.770
1	1.721	.019	1.685	1.758

Univariate Analysis of Variance-CAST B

Dependent Variable:fecundity

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	1326.540	1	1326.540	129.606	.000
	Error	41.241	4.029	10.235[a]		
Breed	Hypothesis	56.676	4	14.169	106.279	.000
	Error	86.924	652	.133[b]		
CASTB	Hypothesis	.141	1	.141	1.055	.305
	Error	86.924	652	.133[b]		

a. .720 MS(Breed) + .280 MS(Error)

b. MS(Error)

Estimated Marginal Means-CAST B

Dependent Variable: fecundity

CASTB	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.747	.030	1.688	1.806
1	1.711	.018	1.675	1.747

Univariate Analysis of Variance-CAST C

Dependent Variable:fecundity

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	713.382	1	713.382	129.587	.000
	Error	22.711	4.125	5.505[a]		
Breed	Hypothesis	59.316	4	14.829	111.498	.000
	Error	86.715	652	.133[b]		
CASTC	Hypothesis	.350	1	.350	2.628	.105
	Error	86.715	652	.133[b]		

a. .366 MS(Breed) + .634 MS(Error)

b. MS(Error)

Estimated Marginal Means-CAST C

Dependent Variable:fecundity

CASTC	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.713	.016	1.682	1.745
1	1.788	.044	1.701	1.874

Univariate Analysis of Variance-CAST D

Dependent Variable:fecundity

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	51.423	1	51.423	104.379	.000
	Error	3.634	7.377	.493[a]		
Breed	Hypothesis	59.580	4	14.895	111.759	.000
	Error	86.897	652	.133[b]		
CASTD	Hypothesis	.167	1	.167	1.255	.263
	Error	86.897	652	.133[b]		

a. .024 MS(Breed) + .976 MS(Error)

b. MS(Error)

Estimated Marginal Means-CAST D

Dependent Variable:fecundity

CASTD	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.720	.015	1.689	1.750
1	1.927	.185	1.564	2.290

Univariate Analysis of Variance-TLR4 A

Dependent Variable:fecundity

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	1531.703	1	1531.703	153.146	.000
	Error	40.148	4.014	10.002[a]		
Breed	Hypothesis	46.015	4	11.504	86.175	.000
	Error	87.038	652	.133[b]		
TLR4A	Hypothesis	.026	1	.026	.198	.656
	Error	87.038	652	.133[b]		

a. .868 MS(Breed) + .132 MS(Error)

b. MS(Error)

Estimated Marginal Means-TLR4 A

Dependent Variable:fecundity

TLR4A	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.730	.026	1.679	1.782
1	1.716	.019	1.677	1.754

Univariate Analysis of Variance-TLR4 B

Dependent Variable:fecundity

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	1639.524	1	1639.524	139.996	.000
	Error	46.936	4.008	11.711[a]		
Breed	Hypothesis	51.179	4	12.795	95.957	.000
	Error	86.936	652	.133[b]		
TLR4B	Hypothesis	.128	1	.128	.963	.327
	Error	86.936	652	.133[b]		

a. .914 MS(Breed) + .086 MS(Error)

b. MS(Error)

Estimated Marginal Means-TLR4 B

Dependent Variable:fecundity

TLR4B	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.703	.024	1.657	1.750
1	1.735	.021	1.694	1.776

Univariate Analysis of Variance-TLR4 C

Dependent Variable:fecundity

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	1113.716	1	1113.716	119.564	.000
	Error	37.637	4.041	9.315[a]		
Breed	Hypothesis	57.533	4	14.383	109.075	.000
	Error	85.976	652	.132[b]		
TLR4C	Hypothesis	1.088	1	1.088	8.251	.004
	Error	85.976	652	.132[b]		

a. .644 MS(Breed) + .356 MS(Error)

b. MS(Error)

Estimated Marginal Means-TLR4 C

Dependent Variable:fecundity

TLR4C	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.745	.017	1.710	1.779
1	1.638	.033	1.574	1.702

Univariate Analysis of Variance-TLR4 D

Dependent Variable:fecundity

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	747.046	1	747.046	126.608	.000
	Error	24.267	4.113	5.900[a]		
Breed	Hypothesis	59.853	4	14.963	112.211	.000
	Error	86.943	652	.133[b]		
TLR4D	Hypothesis	.121	1	.121	.908	.341
	Error	86.943	652	.133[b]		

a. .389 MS(Breed) + .611 MS(Error)

b. MS(Error)

Estimated Marginal Means-TLR4 D

Dependent Variable:fecundity

TLR4D	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.716	.016	1.684	1.748
1	1.759	.043	1.675	1.842

Appendix I: Statistical analyses for association of longevity and variation in *IGF1R*, *FOXO3*, *CAST* and *TLR4*-flock correction model

Univariate Analysis of Variance-IGF1R A

Dependent Variable:age

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	4240.766	1	4240.766	246.660	.000
	Error	2129.202	123.843	17.193[a]		
IGF1RA	Hypothesis	.533	1	.533	.059	.808
	Error	15150.481	1681	9.013[b]		
farm2	Hypothesis	3233.054	35	92.373	10.249	.000
	Error	15150.481	1681	9.013[b]		

a. .098 MS(farm2) + .902 MS(Error)

b. MS(Error)

Estimated Marginal Means-IGF1R A

Dependent Variable:age

IGF1RA	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	4.981	.456	4.086	5.875
1	5.093	.077	4.942	5.244

Univariate Analysis of Variance-IGF1R B

Dependent Variable:age

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	15231.924	1	15231.924	400.538	.000
	Error	1861.040	48.938	38.029[a]		
farm2	Hypothesis	3236.373	35	92.468	10.259	.000
	Error	15150.853	1681	9.013[b]		
IGF1RB	Hypothesis	.161	1	.161	.018	.894
	Error	15150.853	1681	9.013[b]		

a. .348 MS(farm2) + .652 MS(Error)

b. MS(Error)

Estimated Marginal Means-IGF1R B

Dependent Variable:age

IGF1RB	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	5.093	.081	4.935	5.251
1	5.060	.234	4.602	5.518

Univariate Analysis of Variance-IGF1R C

Dependent Variable:age

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	20533.721	1	20533.721	477.097	.000
	Error	1935.179	44.963	43.039[a]		
farm2	Hypothesis	3052.158	35	87.205	9.705	.000
	Error	15105.241	1681	8.986[b]		
IGF1RC	Hypothesis	45.773	1	45.773	5.094	.024
	Error	15105.241	1681	8.986[b]		

a. .435 MS(farm2) + .565 MS(Error)

b. MS(Error)

Estimated Marginal Means-IGF1R C

Dependent Variable:age

IGF1RC	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	5.022	.082	4.862	5.183
1	5.518	.204	5.117	5.919

Univariate Analysis of Variance-FOXO3 A

Dependent Variable:age

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	29154.447	1	29154.447	459.398	.000
	Error	2635.789	41.533	63.462[a]		
FOXO3A	Hypothesis	2.895	1	2.895	.336	.562
	Error	14554.640	1692	8.602[b]		
farm2	Hypothesis	3396.817	38	89.390	10.392	.000
	Error	14554.640	1692	8.602[b]		

a. .679 MS(farm2) + .321 MS(Error)

b. MS(Error)

Estimated Marginal Means-FOXO3 A

Dependent Variable:age

FOXO3A	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	5.067	.156	4.762	5.372
1	4.959	.089	4.785	5.133

Univariate Analysis of Variance-FOXO3 B

Dependent Variable:age

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	35662.995	1	35662.995	470.613	.000
	Error	2979.585	39.319	75.780[a]		
farm2	Hypothesis	3327.103	38	87.555	10.185	.000
	Error	14545.660	1692	8.597[b]		
FOXO3B	Hypothesis	11.875	1	11.875	1.381	.240
	Error	14545.660	1692	8.597[b]		

a. .851 MS(farm2) + .149 MS(Error)

b. MS(Error)

Estimated Marginal Means-FOXO3 B

Dependent Variable:age

FOXO3B	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	5.063	.098	4.871	5.255
1	4.862	.130	4.607	5.117

Univariate Analysis of Variance-FOXO3 C

Dependent Variable:age

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	2307.142	1	2307.142	179.430	.000
	Error	3415.849	265.656	12.858[a]		
farm2	Hypothesis	3400.896	38	89.497	10.402	.000
	Error	14557.134	1692	8.604[b]		
FOXO3C	Hypothesis	.401	1	.401	.047	.829
	Error	14557.134	1692	8.604[b]		

a. .053 MS(farm2) + .947 MS(Error)

b. MS(Error)

Estimated Marginal Means-FOXO3 C

Dependent Variable:age

FOXO3C	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	4.986	.074	4.840	5.132
1	5.119	.611	3.920	6.318

Univariate Analysis of Variance-FOXO3 D

Dependent Variable:age

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	29994.489	1	29994.489	458.047	.000
	Error	2674.439	40.841	65.483[a]		
farm2	Hypothesis	3289.611	38	86.569	10.089	.000
	Error	14518.811	1692	8.581[b]		
FOXO3D	Hypothesis	38.724	1	38.724	4.513	.034
	Error	14518.811	1692	8.581[b]		

a. .730 MS(farm2) + .270 MS(Error)

b. MS(Error)

Estimated Marginal Means-FOXO3 D

Dependent Variable:age

FOXO3D	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	5.102	.091	4.923	5.281
1	4.714	.149	4.422	5.005

Univariate Analysis of Variance-FOXO3 E

Dependent Variable:age

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	5635.803	1	5635.803	285.960	.000
	Error	1914.089	97.121	19.708[a]		
farm2	Hypothesis	3424.930	38	90.130	10.477	.000
	Error	14555.940	1692	8.603[b]		
FOXO3E	Hypothesis	1.595	1	1.595	.185	.667
	Error	14555.940	1692	8.603[b]		

a. .136 MS(farm2) + .864 MS(Error)

b. MS(Error)

Estimated Marginal Means-FOXO3 E

Dependent Variable:age

FOXO3E	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	4.995	.076	4.846	5.143
1	4.828	.378	4.088	5.569

Univariate Analysis of Variance-FOXO3 F

Dependent Variable:age

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	1599.509	1	1599.509	144.147	.000
	Error	5641.158	508.378	11.096[a]		
farm2	Hypothesis	3428.082	38	90.213	10.496	.000
	Error	14542.581	1692	8.595[b]		
FOXO3F	Hypothesis	14.954	1	14.954	1.740	.187
	Error	14542.581	1692	8.595[b]		

a. .031 MS(farm2) + .969 MS(Error)

b. MS(Error)

Estimated Marginal Means-FOXO3 F

Dependent Variable:age

FOXO3F	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	4.978	.074	4.832	5.123
1	6.046	.806	4.466	7.626

Univariate Analysis of Variance-FOXO3 G

Dependent Variable:age

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	2143.488	1	2143.488	177.621	.000
	Error	4111.062	340.665	12.068[a]		
farm2	Hypothesis	3443.545	38	90.620	10.544	.000
	Error	14542.042	1692	8.595[b]		
FOXO3G	Hypothesis	15.493	1	15.493	1.803	.180
	Error	14542.042	1692	8.595[b]		

a. .042 MS(farm2) + .958 MS(Error)

b. MS(Error)

Estimated Marginal Means-FOXO3 G

Dependent Variable:age

FOXO3G	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	4.978	.074	4.832	5.124
1	5.901	.684	4.559	7.242

Univariate Analysis of Variance-CAST A

Dependent Variable:age

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	38583.636	1	38583.636	472.975	.000
	Error	2936.629	35.998	81.577[a]		
farm2	Hypothesis	3222.198	35	92.063	10.203	.000
	Error	15239.921	1689	9.023[b]		
CASTA	Hypothesis	.253	1	.253	.028	.867
	Error	15239.921	1689	9.023[b]		

a. .874 MS(farm2) + .126 MS(Error)

b. MS(Error)

Estimated Marginal Means-CAST A

Dependent Variable:age

CASTA	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	5.085	.125	4.839	5.330
1	5.112	.100	4.917	5.307

Univariate Analysis of Variance-CAST B

Dependent Variable:age

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	30364.011	1	30364.011	457.527	.000
	Error	2538.002	38.243	66.366[a]		
farm2	Hypothesis	3262.545	35	93.216	10.331	.000
	Error	15239.098	1689	9.023[b]		
CASTB	Hypothesis	1.075	1	1.075	.119	.730
	Error	15239.098	1689	9.023[b]		

a. .681 MS(farm2) + .319 MS(Error)

b. MS(Error)

Estimated Marginal Means-CAST B

Dependent Variable:age

CASTB	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	5.149	.159	4.838	5.460
1	5.084	.091	4.906	5.262

Univariate Analysis of Variance-CAST C

Dependent Variable:age

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	19330.738	1	19330.738	457.373	.000
	Error	1926.898	45.591	42.265[a]		
farm2	Hypothesis	3095.498	35	88.443	9.811	.000
	Error	15225.558	1689	9.015[b]		
CASTC	Hypothesis	14.616	1	14.616	1.621	.203
	Error	15225.558	1689	9.015[b]		

a. .419 MS(farm2) + .581 MS(Error)

b. MS(Error)

Estimated Marginal Means-CAST C

Dependent Variable:age

CASTC	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	5.065	.081	4.906	5.224
1	5.349	.209	4.939	5.758

Univariate Analysis of Variance-CAST D

Dependent Variable:age

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	958.162	1	958.162	88.696	.000
	Error	8023.565	742.730	10.803[a]		
farm2	Hypothesis	3261.127	35	93.175	10.326	.000
	Error	15240.091	1689	9.023[b]		
CASTD	Hypothesis	.083	1	.083	.009	.924
	Error	15240.091	1689	9.023[b]		

a. .021 MS(farm2) + .979 MS(Error)

b. MS(Error)

Estimated Marginal Means-CAST D

Dependent Variable:age

CASTD	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	5.101	.076	4.951	5.250
1	5.197	.997	3.241	7.152

Univariate Analysis of Variance-TLR4 A

Dependent Variable:age

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	39474.894	1	39474.894	470.032	.000
	Error	3008.560	35.823	83.983[a]		
farm2	Hypothesis	3255.702	35	93.020	10.309	.000
	Error	15240.100	1689	9.023[b]		
TLR4A	Hypothesis	.073	1	.073	.008	.928
	Error	15240.100	1689	9.023[b]		

a. .892 MS(farm2) + .108 MS(Error)

b. MS(Error)

Estimated Marginal Means-TLR4 A

Dependent Variable:age

TLR4A	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	5.092	.125	4.848	5.337
1	5.108	.105	4.902	5.313

Univariate Analysis of Variance-TLR4 B

Dependent Variable:age

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	39396.623	1	39396.623	468.488	.000
	Error	3011.535	35.812	84.093[a]		
farm2	Hypothesis	3255.928	35	93.027	10.317	.000
	Error	15229.416	1689	9.017[b]		
TLR4B	Hypothesis	10.758	1	10.758	1.193	.275
	Error	15229.416	1689	9.017[b]		

a. .894 MS(farm2) + .106 MS(Error)

b. MS(Error)

Estimated Marginal Means-TLR4 B

Dependent Variable:age

TLR4B	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	4.997	.122	4.758	5.236
1	5.178	.103	4.975	5.381

Univariate Analysis of Variance-TLR4 C

Dependent Variable:age

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	30410.816	1	30410.816	446.529	.000
	Error	2584.958	37.956	68.105[a]		
farm2	Hypothesis	3270.576	35	93.445	10.363	.000
	Error	15230.407	1689	9.017[b]		
TLR4C	Hypothesis	9.767	1	9.767	1.083	.298
	Error	15230.407	1689	9.017[b]		

a. .700 MS(farm2) + .300 MS(Error)

b. MS(Error)

Estimated Marginal Means-TLR4 C

Dependent Variable:age

TLR4C	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	5.152	.090	4.975	5.329
1	4.962	.154	4.660	5.264

Univariate Analysis of Variance-TLR4 D

Dependent Variable:age

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	14931.311	1	14931.311	402.517	.000
	Error	1837.263	49.529	37.095[a]		
farm2	Hypothesis	3176.882	35	90.768	10.061	.000
	Error	15237.548	1689	9.022[b]		
TLR4D	Hypothesis	2.626	1	2.626	.291	.590
	Error	15237.548	1689	9.022[b]		

a. .343 MS(farm2) + .657 MS(Error)

b. MS(Error)

Estimated Marginal Means-TLR4 D

Dependent Variable:age

tlr4d	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	5.117	.082	4.957	5.277
1	4.979	.238	4.512	5.447

Appendix J: Statistical analyses for association of fecundity and variation in *IGF1R*, *FOXO3*, *CAST* and *TLR4*-flock correction model

Univariate Analysis of Variance-IGF1R A

Dependent Variable:fecundity

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	171.578	1	171.578	540.164	.000
	Error	19.457	61.254	.318[a]		
IGF1RA	Hypothesis	.004	1	.004	.043	.835
	Error	62.751	620	.101[b]		
farm2	Hypothesis	82.207	31	2.652	26.201	.000
	Error	62.751	620	.101[b]		

a. .085 MS(farm2) + .915 MS(Error)

b. MS(Error)

Estimated Marginal Means-IGF1R A

Dependent Variable:fecundity

IGF1RA	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.754	.085	1.588	1.920
1	1.772	.013	1.747	1.797

Univariate Analysis of Variance-IGF1R B

Dependent Variable:fecundity

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	689.829	1	689.829	693.859	.000
	Error	35.310	35.517	.994[a]		
farm2	Hypothesis	82.219	31	2.652	26.321	.000
	Error	62.474	620	.101[b]		
IGF1RB	Hypothesis	.282	1	.282	2.795	.095
	Error	62.474	620	.101[b]		

a. .350 MS(farm2) + .650 MS(Error)

b. MS(Error)

Estimated Marginal Means-IGF1R B

Dependent Variable:fecundity

IGF1RB	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.779	.014	1.753	1.806
1	1.707	.040	1.628	1.787

Univariate Analysis of Variance-IGF1R C

Dependent Variable:fecundity

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	974.766	1	974.766	746.277	.000
	Error	43.993	33.681	1.306[a]		
farm2	Hypothesis	81.796	31	2.639	26.085	.000
	Error	62.716	620	.101[b]		
IGF1RC	Hypothesis	.039	1	.039	.386	.535
	Error	62.716	620	.101[b]		

a. .475 MS(farm2) + .525 MS(Error)

b. MS(Error)

Estimated Marginal Means-IGF1R C

Dependent Variable:fecundity

IGF1RC	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.768	.014	1.740	1.795
1	1.791	.034	1.724	1.858

Univariate Analysis of Variance-FOXO3 A

Dependent Variable:fecundity

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	1423.348	1	1423.348	773.041	.000
	Error	59.065	32.079	1.841[a]		
FOXO3A	Hypothesis	.179	1	.179	1.722	.190
	Error	62.260	598	.104[b]		
farm2	Hypothesis	80.172	31	2.586	24.840	.000
	Error	62.260	598	.104[b]		

a. .700 MS(farm2) + .300 MS(Error)

b. MS(Error)

Estimated Marginal Means-FOXO3 A

Dependent Variable:fecundity

FOXO3A	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.831	.029	1.775	1.887
1	1.786	.016	1.753	1.818

Univariate Analysis of Variance-FOXO3 B

Dependent Variable:fecundity

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	1630.108	1	1630.108	775.354	.000
	Error	66.433	31.599	2.102[a]		
farm2	Hypothesis	79.869	31	2.576	24.682	.000
	Error	62.421	598	.104[b]		
FOXO3B	Hypothesis	.018	1	.018	.176	.675
	Error	62.421	598	.104[b]		

a. .808 MS(farm2) + .192 MS(Error)

b. MS(Error)

Estimated Marginal Means-FOXO3 B

Dependent Variable:fecundity

FOXO3B	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.794	.017	1.760	1.827
1	1.807	.026	1.757	1.857

Univariate Analysis of Variance-FOXO3 C

Dependent Variable:fecundity

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	100.259	1	100.259	443.688	.000
	Error	21.640	95.764	.226[a]		
farm2	Hypothesis	83.856	31	2.705	25.938	.000
	Error	62.365	598	.104[b]		
FOXO3C	Hypothesis	.075	1	.075	.716	.398
	Error	62.365	598	.104[b]		

a. .047 MS(farm2) + .953 MS(Error)

b. MS(Error)

Estimated Marginal Means-FOXO3 C

Dependent Variable fecundity

FOXO3C	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.797	.013	1.771	1.823
1	1.898	.118	1.665	2.130

Univariate Analysis of Variance-FOXO3 D

Dependent Variable:fecundity

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	1501.437	1	1501.437	799.648	.000
	Error	59.870	31.886	1.878[a]		
farm2	Hypothesis	76.710	31	2.475	23.709	.000
	Error	62.414	598	.104[b]		
FOXO3D	Hypothesis	.026	1	.026	.245	.621
	Error	62.414	598	.104[b]		

a. .748 MS(farm2) + .252 MS(Error)

b. MS(Error)

Estimated Marginal Means-FOXO3 D

Dependent Variable:fecundity

FOXO3D	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.803	.017	1.770	1.836
1	1.786	.027	1.733	1.840

Univariate Analysis of Variance-FOXO3 E

Dependent Variable:fecundity

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	238.313	1	238.313	584.205	.000
	Error	21.014	51.514	.408[a]		
farm2	Hypothesis	83.536	31	2.695	25.809	.000
	Error	62.438	598	.104[b]		
FOXO3E	Hypothesis	.001	1	.001	.014	.905
	Error	62.438	598	.104[b]		

a. .117 MS(farm2) + .883 MS(Error)

b. MS(Error)

Estimated Marginal Means-FOXO3 E

Dependent Variable:fecundity

FOXO3E	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.798	.014	1.771	1.825
1	1.807	.075	1.660	1.954

Univariate Analysis of Variance-FOXO3 F

Dependent Variable:fecundity

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	101.982	1	101.982	456.534	.000
	Error	21.717	97.219	.223[a]		
farm2	Hypothesis	83.880	31	2.706	26.003	.000
	Error	62.226	598	.104[b]		
FOXO3F	Hypothesis	.214	1	.214	2.055	.152
	Error	62.226	598	.104[b]		

a. .046 MS(farm2) + .954 MS(Error)

b. MS(Error)

Estimated Marginal Means-FOXO3 F

Dependent Variable:fecundity

FOXO3F	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.796	.013	1.770	1.822
1	1.968	.120	1.734	2.203

Univariate Analysis of Variance-FOXO3 G

Dependent Variable:fecundity

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	118.933	1	118.933	475.653	.000
	Error	20.632	82.513	.250[a]		
farm2	Hypothesis	84.071	31	2.712	26.012	.000
	Error	62.345	598	.104[b]		
FOXO3G	Hypothesis	.094	1	.094	.904	.342
	Error	62.345	598	.104[b]		

a. .056 MS(farm2) + .944 MS(Error)

b. MS(Error)

Estimated Marginal Means-FOXO3 G

Dependent Variable:fecundity

FOXO3G	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.797	.013	1.771	1.823
1	1.900	.108	1.688	2.113

Univariate Analysis of Variance-CAST A

Dependent Variable:fecundity

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	1876.633	1	1876.633	793.623	.000
	Error	73.834	31.224	2.365[a]		
farm2	Hypothesis	79.716	31	2.571	25.314	.000
	Error	63.489	625	.102[b]		
CASTA	Hypothesis	.006	1	.006	.059	.808
	Error	63.489	625	.102[b]		

a. .916 MS(farm2) + .084 MS(Error)

b. MS(Error)

Estimated Marginal Means-CAST A

Dependent Variable:fecundity

CASTA	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.775	.021	1.733	1.817
1	1.768	.017	1.735	1.801

Univariate Analysis of Variance-CAST B

Dependent Variable:fecundity

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	1431.254	1	1431.254	782.442	.000
	Error	58.671	32.074	1.829[a]		
farm2	Hypothesis	80.142	31	2.585	25.462	.000
	Error	63.458	625	.102[b]		
CASTB	Hypothesis	.037	1	.037	.364	.546
	Error	63.458	625	.102[b]		

a. .696 MS(farm2) + .304 MS(Error)

b. MS(Error)

Estimated Marginal Means-CAST B

Dependent Variable:fecundity

CASTB	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.786	.027	1.732	1.839
1	1.766	.015	1.736	1.796

Univariate Analysis of Variance-CAST C

Dependent Variable:fecundity

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	778.239	1	778.239	740.007	.000
	Error	36.941	35.126	1.052[a]		
farm2	Hypothesis	82.712	31	2.668	26.336	.000
	Error	63.319	625	.101[b]		
CASTC	Hypothesis	.176	1	.176	1.733	.189
	Error	63.319	625	.101[b]		

a. .370 MS(farm2) + .630 MS(Error)

b. MS(Error)

Estimated Marginal Means-CAST C

Dependent Variable:fecundity

CASTC	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.765	.014	1.738	1.791
1	1.820	.039	1.743	1.897

Univariate Analysis of Variance-CAST D

Dependent Variable:fecundity

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	50.222	1	50.222	320.553	.000
	Error	31.348	200.091	.157[a]		
farm2	Hypothesis	83.189	31	2.684	26.501	.000
	Error	63.288	625	.101[b]		
CASTD	Hypothesis	.207	1	.207	2.045	.153
	Error	63.288	625	.101[b]		

a. .021 MS(farm2) + .979 MS(Error)

b. MS(Error)

Estimated Marginal Means-CAST D

Dependent Variable:fecundity

CASTD	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.770	.013	1.745	1.794
1	2.013	.169	1.680	2.345

Univariate Analysis of Variance-TLR4 A

Dependent Variable:fecundity

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	1907.485	1	1907.485	905.815	.000
	Error	65.716	31.207	2.106[a]		
farm2	Hypothesis	69.900	31	2.255	22.315	.000
	Error	63.153	625	.101[b]		
TLR4A	Hypothesis	.342	1	.342	3.386	.066
	Error	63.153	625	.101[b]		

a. .931 MS(farm2) + .069 MS(Error)

b. MS(Error)

Estimated Marginal Means-TLR4 A

Dependent Variable:fecundity

TLR4A	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.804	.022	1.761	1.848
1	1.748	.018	1.712	1.783

Univariate Analysis of Variance-TLR4 B

Dependent Variable:fecundity

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	1890.088	1	1890.088	839.138	.000
	Error	70.266	31.196	2.252[a]		
farm2	Hypothesis	74.838	31	2.414	23.845	.000
	Error	63.277	625	.101[b]		
TLR4B	Hypothesis	.218	1	.218	2.153	.143
	Error	63.277	625	.101[b]		

a. .930 MS(farm2) + .070 MS(Error)

b. MS(Error)

Estimated Marginal Means-TLR4 B

Dependent Variable fecundity

TLR4B	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.745	.022	1.703	1.788
1	1.789	.018	1.754	1.823

Univariate Analysis of Variance-TLR4 C

Dependent Variable:fecundity

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	1281.617	1	1281.617	760.029	.000
	Error	54.626	32.395	1.686[a]		
farm2	Hypothesis	80.254	31	2.589	25.579	.000
	Error	63.255	625	.101[b]		
TLR4C	Hypothesis	.240	1	.240	2.368	.124
	Error	63.255	625	.101[b]		

a. .637 MS(farm2) + .363 MS(Error)

b. MS(Error)

Estimated Marginal Means-TLR4 C

Dependent Variable:fecundity

TLR4C	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.783	.015	1.754	1.813
1	1.731	.029	1.673	1.788

Univariate Analysis of Variance-TLR4 D

Dependent Variable:fecundity

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	714.333	1	714.333	711.495	.000
	Error	35.659	35.517	1.004[a]		
farm2	Hypothesis	83.301	31	2.687	26.451	.000
	Error	63.495	625	.102[b]		
TLR4D	Hypothesis	.000	1	.000	.002	.961
	Error	63.495	625	.102[b]		

a. .349 MS(farm2) + .651 MS(Error)

b. MS(Error)

Estimated Marginal Means-TLR4 D

Dependent Variable:fecundity

TLR4D	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.771	.014	1.744	1.798
1	1.773	.041	1.692	1.854

Appendix K: Statistical analyses for association of birth weight and variation in *CAST*

CAST versus Birth weight - Results summary Jan 2008

Trial flock = Hugh Taylor 2006 born lambs

Birthweight:

Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation
Birth Weight	519	2.75	8.75	5.6830	.92711
Valid N (listwise)	519				

Descriptive Statistics

Dependent Variable: Birth Weight

RAM ID	Mean	Std. Deviation	N
Banklea 217/00	5.9875	1.22333	20
Braebank 67/03	5.5577	.69032	26
Doughboy 45/04	5.8378	.88234	37
Glenleith 25/02	5.7273	.87358	33
Glenleith 252/04	5.7685	.90119	27
Hermiston 22/04	5.7907	1.09236	43
Lammerlaw 77/04	5.8214	.93255	21
Leeds Lodge 26/03	5.7400	1.02693	25
Longridge 626/02	5.4143	1.01631	35
Mana 83/04	5.7100	.94285	25
Mana 90/01	5.6111	.85240	36
Offord 414/01	5.6889	.79264	45
Snowlea 192/02	6.0078	.94716	32
Sudeley 102/04	5.7321	.99519	28
Tanlet 547/02	5.2778	.84163	27
Totaranui 376/02	5.6061	.90362	33
Waidale 618/04	5.3558	.68983	26
Total	5.6830	.92711	519

Half-sib analyses

The following CAST genotypes were deduced from the progeny genotypes

Sire	CAST Genotype
Banklea217/00	**AB or BC**
Braebank 67/03	**BB**
Doughboy 45/04	**BB**
Glenleith 25/02	**BB**
Glenleith 252/04	**BC**
Hermiston 22/04	**AB**
Lammerlaw 77/04	**BC**
Leeds Lodge 26/03	**AB or BC**
Longridge 626/02	**BC**
Mana 83/04	**AB**
Offord 414/01	**BB**
Snowlea 192/02	**BB**
Sudeley 102/04	**BB**
Tanlet 547/02	**BB**
Totaranui 376/02	**BB**
Waidale 618/04	**BB**

Glenleith 252/04, Hermiston 22/04, Lammerlaw 77/04, Longridge 626/02, Mana 83/04 were analysed. No significant sire allele effect were detected. This is not surprising as in each case at least one of the sire allele groups had $n < 5$

Analyses of the Pooled Data

Testing the independent effects of each of the CAST alleles.

For each CAST allele an ANOVA was performed using the significance level of $\alpha = 0.05$ to assess the effect of allele presence, sire, and birth-rank (whether the animal was a single or multiple) as well as the interactions of these factors on birthweight ie a full factorial model was employed. If there were no significant interactions, these terms were removed from the ANOVA model and only the main effects tested.

1. CASTa

Between-Subjects Factors

		N
RAM CODE	1	16
	2	22
	3	36
	4	30
	5	24
	6	41
	7	17
	8	24
	9	31
	10	20
	11	30
	12	40
	13	32
	14	24
	15	24
	16	30
	17	21
SM	m	365
	s	97
CASTa	0	325
	1	137

Full factorial model: No sire effect on birthweight was detected (P = 0.622). Both birth-rank and the presence of CASTa had a significant effect on birthweight (P≤0.001 and P = 0.019, repectively). As expected single born lambs had a heavier birthweight than there multiple born counterparts (singles 5.496 ± 0.054kg; multiples 6.580 ± 0.106kg). Those lambs possessing the CASTa allele were also heavier, having a mean birthweight of 6.084 ± 0.094kg, while those lambs that did not possess the CASTa allele had a mean birthweight of 5.914 ± 0.068kg. A significant interaction between birthrank and CASTa was also detected (P = 0.008).

SM * CASTa

Dependent Variable: Birth Weight

SM	CASTa	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
m	0	5.531	.055	5.424	5.638
	1	5.452	.091	5.273	5.631
s	0	6.334	.129	6.080	6.587
	1	6.910	.196	6.525	7.296

To investigate this interaction further the file was split by birth-rank and each rank analysed independently. Obviously birth-rank was removed from the ANOVA model.

Muliples: No sire effect on birthweight was detected (P = 0.360). The presence of CASTa had no effect on birthweight (P = 0.597). An interaction between sire and CASTa was detected (P = 0.029)

RAM CODE * CASTa(a)

Dependent Variable: Birth Weight

RAM CODE	CASTa	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
1	0	5.833	.273	5.296	6.371
	1	5.583	.473	4.652	6.514
2	0	5.547	.205	5.144	5.950
	1	5.333	.473	4.402	6.264
3	0	5.779	.199	5.388	6.170
	1	5.614	.247	5.128	6.100
4	0	5.234	.205	4.831	5.637
	1	6.083	.335	5.425	6.742
5	0	5.544	.199	5.153	5.935
	1	5.833	.473	4.902	6.764
6	0	5.203	.205	4.800	5.606
	1	5.643	.219	5.212	6.074
7	0	5.375	.259	4.865	5.885
	1	5.500	.580	4.360	6.640
8	0	5.222	.273	4.685	5.760
	1	6.000	.273	5.463	6.537
9	0	5.382	.199	4.991	5.773
	1	5.125	.259	4.615	5.635
10	0	6.179	.310	5.569	6.788
	1	5.437	.237	4.972	5.903
11	0	5.500	.193	5.120	5.880
	1	4.833	.473	3.902	5.764
12	0	5.404	.161	5.088	5.720
	1	5.650	.367	4.929	6.371
13	0	5.908	.188	5.538	6.278
	1	4.583	.473	3.652	5.514
14	0	5.827	.227	5.380	6.274
	1	5.444	.273	4.907	5.982
15	0	5.233	.212	4.817	5.650
	1	4.800	.367	4.079	5.521
16	0	5.353	.199	4.962	5.744
	1	5.700	.367	4.979	6.421
17	0	5.385	.227	4.937	5.832
	1	5.786	.310	5.176	6.395

a SM = m

Singles: No sire effect on birthweight was detected (P = 0.813). The presence of CASTa had a significant effect on birthweight (P = 0.007) with those animals possessing the CASTa allele having a mean birthweight of 6.890 ± 0.183 kg and while their counterparts not possessing the CASTa allele had a mean birthweight of 6.328 ± 0.130 kg. A 2-way interaction between sire and CASTa was not detected (P = 0.340)

2. CASTb

Between-Subjects Factors

		N
RAM	1	16
CODE	2	22
	3	36
	4	30
	5	24
	6	41
	7	17
	8	24
	9	31
	10	20
	11	30
	12	40
	13	32
	14	24
	15	24
	16	30
	17	21
SM	m	365
	s	97
CASTb	0	19
	1	443

Full factorial model: As expected (given the CASTa results) no sire effect on birthweight was detected ($P = 0.574$) whereas a significant effect of birth-rank on birthweight was observed ($P \leq 0.001$). No effect of the presence of CASTb on birthweight was detected ($P = 0.143$). There were no significant interactions.

Customised model: When the interaction terms were removed from the ANOVA model and only the main effects were tested no sire effect on birthweight was detected ($P = 0.456$), a significant effect of birth-rank on birthweight was observed ($P \leq 0.001$) and there was a strong tendency for those animals that possessed the CASTb allele to have a heavier birthweight than those that did not ($P = 0.055$; mean birthweight CASTb present 6.036 ± 0.0.051 kg, absent 5.638 ± 0.204kg).

3. CASTc

Between-Subjects Factors

		N
RAM CODE	1	16
	2	22
	3	36
	4	30
	5	24
	6	41
	7	17
	8	24
	9	31
	10	20
	11	30
	12	40
	13	32
	14	24
	15	24
	16	30
	17	21
SM	m	365
	s	97
CASTc	0	385
	1	77

Full factorial model: As expected (given the CASTa results) no sire effect on birthweight was detected (P = 0.586) whereas a significant effect of birth-rank on birthweight was observed (P ≤ 0.001). A significant effect of the presence of CASTc on birthweight was detected (P ≤ 0.001)with those animals possessing the CASTc allele having a mean birthweight of 5.529 ± 0.122 kg while those that did not possess the CASTc allele had a heavier mean birthweight of 6.138 ± 0.069kg . There were no significant interactions.

Customised model: When the interaction terms were removed from the ANOVA model and only the main effects were tested, no sire effect on birthweight was detected (P = 0.410), whereas a significant effect of birth-rank (P ≤ 0.001) and the presence of the CASTc allele (P = 0.002) on birthweight were detected. Those animals possessing the CASTc allele had a mean birthweight of 5.745 ± 0.102 kg while those that did not possess the CASTc allele had a heavier mean birthweight of 6.079 ± 0.053kg. NOTE: the means quoted are estimated marginal means and therefore change with the ANOVA model used.

Testing the effect of CAST genotype:

An ANOVA was performed using the significance level of $\alpha = 0.05$ to assess the effect of CAST genotype, sire, and birth-rank (whether the animal was a single or multiple) as well as the interactions of these factors on birthweight ie a full factorial model was employed. If there were no significant interactions, these terms were removed from the ANOVA model and only the main effects tested.

Before undertaking this analysis the rarer genotypes were removed from the data set.

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	AA	3	.6	.6	.6
	AB	121	23.2	26.1	26.7
	AC	13	2.5	2.8	29.5
	BB	263	50.5	56.7	86.2
	BC	61	11.7	13.1	99.4
	CC	3	.6	.6	100.0
	Total	464	89.1	100.0	
Missing	.	57	10.9		
Total		521	100.0		

Using the data from those lambs that had the genotypes AB, BB or BC:

Between-Subjects Factors

		N
RAM CODE	1	14
	2	22
	3	36
	4	30
	5	22
	6	37
	7	13
	8	24
	9	27
	10	17
	11	30
	12	40
	13	32
	14	24
	15	24
	16	30
	17	21
SM	m	349
	s	94
CASTlamb	AB	121
	BB	261
	BC	61

Full factorial model: No sire effect on birthweight was detected ($P = 0.367$) whereas a significant effect of birth-rank on birthweight was observed ($P \leq 0.001$). A significant effect of CAST genotype on birthweight was also detected ($P \leq 0.004$). There were no significant interactions.

Estimates

Dependent Variable: Birth Weight

CASTlamb	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
AB	6.161[a]	.100	5.963	6.358
BB	6.017[a]	.076	5.867	6.167
BC	5.591[a]	.130	5.336	5.846

a. Based on modified population marginal mean.

Pairwise Comparisons

Dependent Variable: Birth Weight

(I) CASTlamb	(J) CASTlamb	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
AB	BB	.144[b,c]	.126	.255	-.104	.391
	BC	.570*[,b,c]	.164	.001	.247	.892
BB	AB	-.144[b,c]	.126	.255	-.391	.104
	BC	.426*[,b,c]	.150	.005	.130	.722
BC	AB	-.570*[,b,c]	.164	.001	-.892	-.247
	BB	-.426*[,b,c]	.150	.005	-.722	-.130

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

a. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

b. An estimate of the modified population marginal mean (I).

c. An estimate of the modified population marginal mean (J).

The above tables show a significant difference between the mean birthweight of those animals with the AB or BB genotypes when compared to those with the BC genotype (LSD, P = 0.001 and P = 0.005, respectively).

Customised model: When the interaction terms were removed from the ANOVA model and only the main effects were tested, no sire effect on birthweight was detected (P = 0.430), whereas a significant effect of birth-rank (P ≤ 0.001) and CAST genotype (P = 0.019) on birthweight were detected.

Estimates

Dependent Variable: Birth Weight

CASTlamb	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
AB	6.169	.084	6.005	6.333
BB	6.039	.062	5.917	6.160
BC	5.785	.112	5.565	6.005

Pairwise Comparisons

Dependent Variable: Birth Weight

(I) CASTlamb	(J) CASTlamb	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
AB	BB	.130	.095	.170	-.056	.316
	BC	.384*	.136	.005	.117	.650
BB	AB	-.130	.095	.170	-.316	.056
	BC	.254*	.121	.037	.015	.492
BC	AB	-.384*	.136	.005	-.650	-.117
	BB	-.254*	.121	.037	-.492	-.015

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

a. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

The above tables show a significant difference between the mean birthweight of those animals with the AB or BB genotypes when compared to that of those with the BC genotype (LSD, P = 0.005 and P = 0.037, respectively). The mean birthweight of those animals with the BC CAST genotype were lighter. The mean birthweight for those animals with AB CAST genotypes was not significantly different to those with BB genotypes (LSD, P = 0.170)

Using the data from those lambs that had the genotypes AB, AC, BB or BC:

Between-Subjects Factors

		N
RAM CODE	1	16
	2	22
	3	36
	4	30
	5	23
	6	39
	7	16
	8	24
	9	30
	10	19
	11	30
	12	40
	13	32
	14	24
	15	24
	16	30
	17	21
SM	m	361
	s	95
CASTlamb	AB	121
	AC	13
	BB	261
	BC	61

Full factorial model: No sire effect on birthweight was detected (P = 0.330) whereas a significant effect of birth-rank on birthweight was observed (P ≤ 0.001). A significant effect of CAST genotype on birthweight was also detected (P ≤ 0.001) along with a 2-way interaction between birth-rank and CAST genotype (P = 0.048). There were no other significant interactions.

Estimates

Dependent Variable: Birth Weight

CASTlamb	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
AB	6.161[a]	.101	5.962	6.359
AC	5.244[a]	.247	4.759	5.729
BB	6.017[a]	.077	5.866	6.168
BC	5.591[a]	.131	5.334	5.848

a. Based on modified population marginal mean.

Pairwise Comparisons

Dependent Variable: Birth Weight

(I) CASTlamb	(J) CASTlamb	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
AB	AC	.917*[,b,c]	.267	.001	.392	1.441
	BB	.144[b,c]	.127	.259	-.106	.393
	BC	.570*[,b,c]	.165	.001	.245	.895
AC	AB	-.917*[,b,c]	.267	.001	-1.441	-.392
	BB	-.773*[,b,c]	.258	.003	-1.281	-.265
	BC	-.347[b,c]	.279	.215	-.896	.202
BB	AB	-.144[b,c]	.127	.259	-.393	.106
	AC	.773*[,b,c]	.258	.003	.265	1.281
	BC	.426*[,b,c]	.152	.005	.128	.724
BC	AB	-.570*[,b,c]	.165	.001	-.895	-.245
	AC	.347[b,c]	.279	.215	-.202	.896
	BB	-.426*[,b,c]	.152	.005	-.724	-.128

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

a. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

b. An estimate of the modified population marginal mean (I).

c. An estimate of the modified population marginal mean (J).

The above tables show that the mean birthweight for those animals with the CAST AB genotype is significantly different from those with the AC (LSD, P = 0.001) or BC (LSD, P = 0.001) genotypes, with the latter genotypes having lighter mean birthweights. Likewise the the CAST BB genotype is significantly different from those with the AC (LSD, P = 0.003)or BC (LSD, P = 0.005) genotypes, with the latter genotypes having lighter mean birthweights. The mean birthweight for those animals with AB CAST genotypes was not significantly different to that of those with BB genotypes (LSD, P = 0.259)

Appendix L: Copies of publications arising from this thesis

Byun, S.O., Zhou, H., Forrest, R.H., Frampton, C.M., & Hickford, J.G. (2012). An association between life-span and variation in IGF1R in sheep. *Journal of animal science*, Accepted.

Byun, S.O., Zhou, H., & Hickford, J.G. (2011). Characterisation of genetic variation in the Forkhead box class O3 (FOXO3) in sheep. *DNA and Cell Biology*, 30(7), 449-452.

Byun, S.O., Zhou, H., Frampton, C.M., & Hickford, J.G. (2010) No association between variation in the ovine calpastatin gene and either longevity or fertiltiy in sheep. *Animal Genetics*, 41(2), 223-224.

Byun, S.O., Zhou, H., & Hickford, J.G. (2009). Development of a simple typing method for the ovine Toll-like receptor 4 gene. *Veterinary Immunology and Immunopathology*, 130(3-4), 272-274.

Byun, S.O., Fang, Q., Zhou, H., & Hickford, J.G.(2009). An effective method for silver-staining DNA in large numbers of polyacrylamide gels. *Analytical Biochemistry*, 385(1), 174-175.

Byun, S.O., Zhou, H., & Hickford, J.G.(2009). Haplotypic diversity within the ovine calpastatin (CAST) gene. *Molecular Biotechnology*, 41(2), 133-137.

Byun, S.O., Zhou, H., Forrest, R.H., Frampton, C.M., & Hickford, J.G. (2008). Association of the ovine calpastatin gene with birth weight and growth rate to weaning. *Animal Genetics*, 39(5), 572-573.

Zhou, H., Byun, S.O., Frampton, C.M., Bickerstaffe, R., & Hickford, J.G. (2008). Lack of association between *CAST* SNPs and meat tenderness in sheep. *Animal Genetics*, 39(3), 331-332.

Byun, S.O., Zhou, H., & Hickford, J.G. (2008). Polymorphism of the ovine insulin-like growth factor 1 receptor (IGF1R) gene. *Molecular and Cellular Probes*, 22(2), 131-132.

An association between lifespan and variation in insulin-like growth factor I receptor in sheep

S. O. Byun, R. H. Forrest, C. M. Frampton, H. Zhou and J. G. H. Hickford

J ANIM SCI 2012, 90:2484-2487.
doi: 10.2527/jas.2011-4148

An association between lifespan and variation in insulin-like growth factor I receptor in sheep[1]

S. O. Byun,* R. H. Forrest,† C. M. Frampton,‡ H. Zhou,* and J. G. H. Hickford*[2]

*Faculty of Agriculture and Life Sciences, Lincoln University, Canterbury 7647, New Zealand; †Faculty of Health and Sport Sciences, Eastern Institute of Technology, Napier, New Zealand; and ‡Christchurch School of Medicine and Health Sciences, Christchurch, New Zealand

ABSTRACT: Longevity in livestock is a valuable trait. When productive animals live longer, fewer replacement animals need to be raised. However, selection for longevity is not commonly the focus of breeding programs as direct selection for long-lived breeding stock is virtually impossible until late in the reproductive life of the animal. Additionally the underlying genetic factors or genes associated with longevity are either not known, or not well understood. In humans, there is evidence that IGF 1 receptor (IGF1R) is involved in longevity. Polymorphism in the IGF1R gene has been associated with longevity in a number of species. Recently, 3 alleles of ovine *IGF1R* were identified, but no analysis of the effect of *IGF1R* variation on sheep longevity has been reported. In this study, associations between ovine *IGF1R* variation, longevity and fertility were investigated. Polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP) was used to type *IGF1R* variation in 1,716 New Zealand sheep belonging to 6 breeds and 36 flocks. Ovine *IGF1R C* was associated with age when adjusting for flock (present 5.5 ± 0.2 yr, absent 5.0 ± 0.1 yr, $P = 0.02$). A general linear mixed effects model suggested an association ($P = 0.06$) between age and genotype, when correcting for flock. Pairwise comparison (least significant difference) of specific genotypes revealed the difference to be between *AA* (5.0 ± 0.1 yr) and *AC* (5.6 ± 0.2 yr, $P = 0.02$). A weak negative Pearson correlation between fertility and longevity traits was observed ($r = -0.25$, $P < 0.01$). The finding of an association between variation in *IGF1R* and lifespan in sheep may be useful in prolonging the lifespan of sheep.

Key words: fertility, insulin-like growth factor 1, longevity, polymerase chain reaction-single strand conformational polymorphism, sheep

J. Anim. Sci. 2012.90:2484 2487
doi:10.2527/jas2011-4148

Removed due to copyright

DNA AND CELL BIOLOGY
Volume 30, Number 7, 2011

Pp. 449 452
DOI: 10.1089/dna.2010.1193

Characterization of Genetic Variation in the Forkhead Box Class O3 Gene (*FOXO3*) in Sheep

Seung O.K. Byun, Huitong Zhou, and Jon G.H. Hickford

FOXO3 is a member of the Forkhead box class O (FOXO) transcription factor family that modulates the transcription of target genes in a variety of cellular functions in lower animals to mammals. It has been implicated in the attenuation of insulin-signaling pathways, including having a potential role in lifespan control in rodents and invertebrates. Recent studies also provide some evidence for an association between ageing, human longevity, and variation in the FOXO3 gene. To date there has been no study on variation in ovine *FOXO3* or its relationship to longevity in sheep. In this study, we investigated the variation in exon 2 of ovine *FOXO3*. This exon encompasses the DNA-binding domain, which is the key regulator of transcription activity in the target genes. To enable investigation of the entire exon 2 of the gene, four overlapping fragments were amplified and analyzed by polymerase chain reaction–single-strand conformational polymorphism and DNA sequencing. Seven haplotypes with 10 nucleotide substitutions were identified across the 1422-bp exon 2 fragment. One of these substitutions was predicted to produce an amino acid change (p.M201V). The variation identified in ovine *FOXO3* may have an impact on both the structure and function of ovine FOXO3 and, consequently, affect the longevity of sheep.

Introduction

FOXO1 (FKHR), FOXO3 (FKHRL1), FOXO4 (AFX), and FOXO6 all belong to transcription factors of the Forkhead box O (FOXO) class, which are characterized by having a conserved DNA-binding domain termed the Forkhead box (FOX) (Carlsson and Mahlapuu, 2002). FOXO3 activates the transcription of many genes, including insulin and other growth-related genes (Daitoku and Fukamizu, 2007). In the helminth model organism *Caenorhabditis elegans* and other species, there is evidence that FOXO3 is a major target of insulin-like signaling and that it is therefore important in the regulation of development, metabolism, and longevity (Murphy, 2006).

Structurally, FOXO3 is a 71-kDa protein containing an evolutionally conserved winged-helix Forkhead (FH) box domain. This comprises the DNA-binding domain (that binds the genes that are under transcriptional control) and three additional conserved regions, called the CR1, CR2, and CR3 domains. The FH domain is composed of three major α-helices (H1 3), two large unstructured wings (W1, W2), and three β-strands (S1 3) (Wang *et al*., 2008). It is well established that the FH domain contains a 110-amino acid motif that functions as a specific DNA-binding site (Carlsson and Mahlapuu, 2002) and it has also been demonstrated that intramolecular association between the CR3 domain and the FH domain further contributes to interaction with DNA (Wang *et al*., 2008). This suggests that the CR3 domain also participates in DNA-binding activity.

Recent studies have revealed that the polymorphism in the human FOXO3 gene is associated with longevity (Willcox *et al*., 2008; Flachsbart *et al*., 2009). However, there is a lack of studies on the variation in this gene in other animal species and whether this variation is associated with longevity. In this study, we screened the entire exon 2 region of ovine *FOXO3* to identify whether genetic variation exists in this critical region of the gene. This exon encodes part of the FH, CR2, and CR3 domains, including the DNA-binding sites of this gene and where DNA sequences are highly conserved across species. If variation exists in this region of the ovine FOXO3 gene, then it might be useful for subsequent genetic studies into longevity in sheep.

Materials and Methods

Sheep and DNA source

In total, 120 New Zealand Romney cross-bred sheep were investigated. Blood samples were collected from these animals onto FTA cards (Whatman BioScience, Middlesex, United Kingdom), and genomic DNA was purified using a two-step washing procedure as described by Zhou *et al*. (2006). Briefly, a 1.2-mm blood disc was incubated with 200 μL of 20 mM NaOH solution for 30 min, and after removal of the NaOH solution, the disc was washed in

Department of Agricultural Sciences, Faculty of Agricultural and Life Sciences, Lincoln University, Christchurch, New Zealand.

Table 1. Primers and Annealing Temperatures for Ovine *FOXO3*

Primers (in pairs)	*Primer sequence (5′ 3′)*	*Expected amplicon size (bp)*	*Annealing temperature for PCR (°C)*
Amplicon 1-up	AACTCTATCCGGCACAACC		
Amplicon 1-dn	GCCAGGATGGGGGACAG	365	62
Amplicon 2-up	AACGCCAGCACAGTCAGC		
Amplicon 2-dn	CTTGTTCTCTTGGATGGTCT	420	64
Amplicon 3-up	GACCGTCGTCTCTGAACTC		
Amplicon 3-dn	TTGGCTGACCCGAGGCTG	400	63
Amplicon 4-up	CATGGGCTTGAGCGACTC		
Amplicon 4-dn	TCCCCTTTCCTCAGTGAGC	376	63

PCR, polymerase chain reaction.

200 μL of 1× TE buffer (10 mM Tris-HCl and 0.1 mM EDTA, pH 8.0).

Polymerase chain reaction primers and amplification

Four sets of polymerase chain reaction (PCR) primers (Table 1) were designed based on the bovine *FOXO3* sequences (GenBank accession no. XM 615634) to putatively amplify the entire second exon of the ovine FOXO3 gene. These were synthesized by Proligo (Boulder, CO).

Amplification was performed in a 20 μL reaction mixture containing the washed genomic DNA on one 1.2-mm punch of FTA paper, 0.25 μM of each primer, 150 μM dNTPs (Eppendorf, Hamburg, Germany), 1.5 mM of $MgCl_2$, 0.5 U of *Taq* DNA polymerase (Qiagen, Hilden, Germany), and 1× reaction buffer. Amplification was carried out in an iCycler (Bio-Rad Laboratories, Hercules, CA) and consisted of denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, the optimized annealing temperature for any given primer pair (Table 1) for 30 s, and 72°C for 30 s, with a final extension step at 72°C for 5 min. Amplicons were visualized by electrophoresis in 1% agarose (Quantum Scientific, Queensland, Australia) gels, using 1× TBE buffer (89 mM Tris, 89 mM boric acid, and 2 mM Na_2EDTA) containing 200 ng/mL of ethidium bromide.

Single-strand conformational polymorphism (SSCP) analysis

A 0.7 μL aliquot of each amplimer was mixed with 7 μL of loading dye (98% formamide, 10 mM EDTA, 0.025% bromophenol blue, and 0.025% xylene-cyanol), and after denaturation at 95°C for 5 min, the samples were rapidly cooled on wet ice and then loaded on to 16×18 cm, 12% acrylamide:bisacrylamide (37.5:1; Bio-Rad Laboratories) gels. Electrophoresis was performed using Protean II xi cells (Bio-Rad Laboratories) at 200 V for 18 h at 25°C in 0.5× TBE buffer. Gels were silver stained according to the method of Byun *et al*. (2009).

Cloning of four fragments covering the entire exon 2 sequence

Sheep DNA samples that produced different PCR-SSCP patterns were selected for amplification using the proofreading enzyme Pwo SuperYield DNA polymerase (Roche Applied Science, Mannheim, Germany), using the conditions previously described. Amplicons were ligated into the pCR4Blunt-TOPO vector (Invitrogen, Carlsbad, CA), and a 2 μL aliquot of the ligation mixture was used to transform competent *Escherichia coli* cells (One Shot INVαF′; Invitrogen), following the manufacturer's instructions. Ten insert positive colonies for each transformation were picked and incubated overnight in Terrific broth (Invitrogen) at 37°C in a shaking rotary incubator (225 rpm).

Clones were screened using a clonal PCR-SSCP approach as previously described (Zhou *et al*., 2008), and only those clones for which the PCR-SSCP patterns matched those of the corresponding genomic DNA were selected for DNA sequencing.

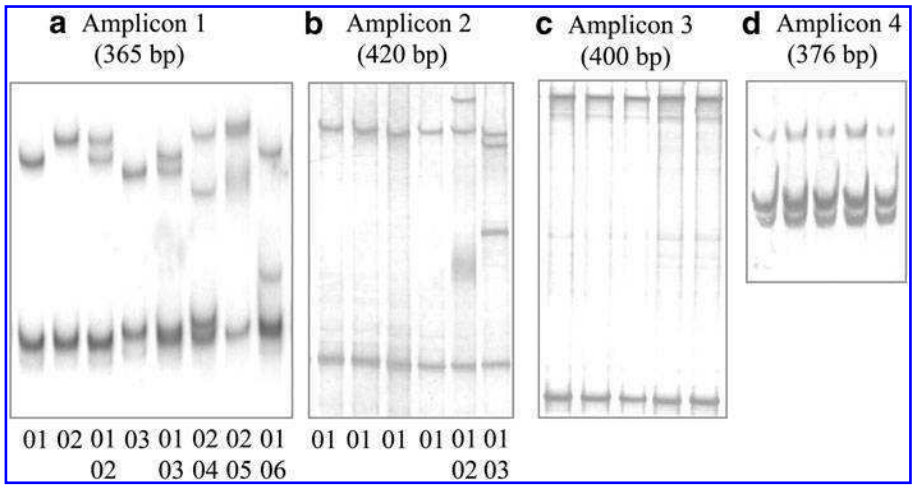


FIG. 1. Polymerase chain reaction single-strand conformational polymorphism of four fragments within exon 2 of ovine *FOXO3*. Six and three unique single-strand conformational polymorphism patterns corresponding to six and three variant sequences (01 06 and 01 03) are shown for amplicons 1 **(a)** and 2 **(b)**, respectively, whereas no polymorphism was detected in amplicons 3 **(c)** and 4 **(d)**.

Table 2. The Seven Different Haplotypes Derived from Amplicons 1 and 2 of Ovine *FOXO3*

Haplotype	*Amplicon 1 (365 bp)* *Amplicon 2 (420 bp)*	*Frequency (%)*[a]
1	01 01	49.0
2	02 01	30.0
3	02 03	2.0
4	03 02	14.0
5	04 01	1.0
6	05 01	3.0
7	06 01	1.0

[a]Rounded frequencies in 120 New Zealand cross bred sheep.

DNA sequencing and sequence analysis

Plasmid DNA was extracted from overnight cultures using a QIAprep Spin Miniprep Kit (Qiagen) and was sequenced in both directions using the M 13-forward and reverse primers. Identical sequences obtained from at least three separate clones from sheep were subjected to further sequence analysis. Sequence alignments, translations, and comparisons were carried out using DNAMAN (Version 5.2.10; Lynnon BioSoft, Vaudreuil, Canada). The BLAST algorithm was used to search the NCBI GenBank (www.ncbi.nlm.nih.gov/) databases for homologous sequences.

Haplotype determination

Haplotypes were determined by screening to find sheep homozygous for any one of the given amplicons and then screening those sheep at the other amplicons to ascertain what sequence variation existed in that region.

Results

To characterize the entire exon 2 region of ovine *FOXO3*, four fragments were amplified using four separate sets of PCR primers (Table 1). Primer sets were designed to produce overlapping amplicons, such that a continuous sequence for the whole of exon 2 could be assembled. The combined fragments produced a 1422-bp sequence covering the entire exon 2 region of ovine *FOXO3*.

Upon SSCP analysis, six and three distinct banding patterns were detected in amplicons 1 and 2, respectively (Fig. 1). No variation was detected in amplicons 3 and 4 (Fig. 1). Either one or two distinct banding patterns were observed for each sheep, which defines homozygous or heterozygous genotypes at the ovine *FOXO3* locus, respectively. Novel variants from amplicons 1 and 2 were sequenced, whereas there was one sequence obtained from amplicons 3 and 4. All the sequences identified shared high homology to the published bovine *FOXO3* sequence (GenBank accession no. XM 615634), suggesting that these sequences represented allelic variants of the ovine FOXO3 gene.

As variation in amplicons 3 and 4 of exon 2 of ovine *FOXO3* was not found, the haplotype was indirectly defined from amplicons 1 and 2 of exon 2 of the gene. Amplicon 1 was used to find homozygous sheep in the population ($n = 120$). Sheep homozygous in amplicon 1 upon SSCP analysis were subjected to further genotyping of amplicon 2 to infer the haplotype over an extended region of the ovine *FOXO3* gene. Seven extended haplotypes were assembled from the four amplicons (Table 2). The seven haplotype sequences detected were named 1 to 7 and deposited into the NCBI GenBank with accession numbers GQ995521 to GQ995527. Sequence analysis revealed 10 nucleotide substitutions in exon 2 (Table 3).

Discussion

In the overall gene structure of *FOXO3*, the entire coding region of the gene is located within exon 1 and exon 2. However, exon 2 encompasses the functional DNA-binding domain of the transcription factor, and therefore, we investigated the genetic variation in this exon. It encodes part of the FH domain, which consists of the DNA-binding site of H3, a long unstructured loop W1, the C-terminal wing W2, and N-terminus of the FH domain. It also encodes conserved domains CR2 and CR3. Recent nuclear magnetic resonance and biochemical studies have demonstrated that the FH and CR domains play a central role in regulating DNA interaction (Tsai *et al*., 2007). Hence, genetic variation in this region of the gene may influence FOXO3 activity and potentially impact both the function of this gene and the genes with which it interacts.

In this study, 10 nucleotide substitutions were identified in the 1422-bp exon 2 fragment assembled (Table 3). One of the substitutions, an A to G substitution at nucleotide position 601, leads to an amino acid change of M to V at

Table 3. Sequence Variation in Exon 2 of the Ovine *FOXO3* Gene

SNP	*Position*[a]	*Haplotype 1*	*Haplotype 2*	*Haplotype 3*	*Haplotype 4*	*Haplotype 5*	*Haplotype 6*	*Haplotype 7*	*Amino acid substitution*
G/A	93	G	G	G	G	A	A	A	
C/T	99	C	C	C	C	C	C	T	
T/C	135	T	T	T	C	T	T	T	
C/T	207	C	T	T	C	C	T	C	
A/G	216	A	A	A	G	A	A	A	
C/T	258	C	C	C	C	C	T	C	
C/T	327	C	C	C	C	C	T	C	
C/A	489	C	C	A	C	C	C	C	
T/C	519	T	T	T	C	T	T	T	
A/G	601	A	A	A	G	A	A	A	M/V

[a]Nucleotide position is related to the first nucleotide of exon 2 of bovine *FOXO3* reference sequence (GenBank accession no. XM 615634). SNP, single nucleotide polymorphism.

position 201 (p.M201V). Substitution at this position has not been found in cattle or any other species to date, even though the FOXO3 protein contains DNA-binding domains that are highly conserved across species (Carlsson and Mahlapuu, 2002). This nonsynonymous substitution in ovine *FOXO3* may be important in determining the activity of the DNA binding-site and, therefore, affect the function of FOXO3.

The rest of substitutions detected in this study are synonymous single-nucleotide polymorphisms and, therefore, potentially functionally neutral (Sharp *et al*., 1995). However, recently, there has been mounting evidence against this line of thought. For example, some reports suggest that synonymous substitutions affect mRNA secondary structure and stability, and hence, they may have functional effects (Parmley *et al*., 2006; Qu *et al*., 2006). The apparently silent polymorphism detected here could therefore also be functionally important.

Mammalian FOXO3 is one of the homologs of daf-16 in *C. elegans*. It is an evolutionarily conserved key regulator of the insulin-like signaling pathway and influences metabolism and lifespan in a variety of species (Murphy, 2006). It has been suggested that there may be a connection between insulin-like growth factor 1 receptor activity (Bonafè *et al*., 2003; Suh *et al*., 2008) and FOXO3 in regulating lifespan.

Recent studies provide evidence that variation in *FOXO3* is associated with human longevity (Willcox *et al*., 2008; Flachsbart *et al*., 2009), and therefore, the genetic variation found in exon 2 of ovine *FOXO3* described in this study may be important in regards to longevity in sheep.

Acknowledgments

Financial support provided by the Lincoln University Gene-Marker Laboratory is gratefully acknowledged. The authors thank Qiang Fang, Guo Yang, Sajee Kunhareang, Hua Gong, Jin Han, and Xueqin Liang for technical assistance.

Disclosure Statement

No competing financial interests exist.

References

Bonafè, M., Barbieri, M., Marcheqiani, F., Olivieri, F., Ragno, E., Giampieri, C., Muqianesi, E., Centurelli, M., Franceschi, C., and Paolisso, G. (2003). Polymorphic variants of insulin like growth factor 1(IGF 1) receptor and phosphoinositide 3 kinase genes affect IGF 1 plasma levels and human longevity: cues for an evolutionarily conserved mechanism of life span con trol. J Clin Endocrinol Metab **88,** 3299 3304.

Byun, S.O., Fang, Q., Zhou, H., and Hickford, J.G. (2009). An effective method for silver staining DNA in large numbers of polyacrylamide gels. Anal Biochem **385,** 174 175.

Carlsson, P., and Mahlapuu, M. (2002). Forkhead transcription factors: key players in development and metabolism. Dev Biol **250,** 1 23.

Daitoku, H., and Fukamizu, A. (2007). FOXO transcription fac tors in the regulatory networks of longevity. J Biochem **141,** 769 774.

Flachsbart, F., Caliebe, A., Kleindorp, R., Blanché, H., von Eller Eberstein, H., Nikolaus, S., Schreiber, S., and Nebel, A. (2009). Association of *FOXO3A* variation with human longevity confirmed in German centenarians. Proc Natl Acad Sci USA **106,** 2700 2705.

Murphy, C.T. (2006). The search for DAF 16/FOXO transcrip tional targets: approaches and discoveries. Exp Gerontol **41,** 910 921.

Parmley, J.L., Chamary, J.V., and Hurst, L.D. (2006). Evidence for purifying selection against synonymous mutations in mam malian exonic splicing enhancers. Mol Biol Evol **23,** 301 309.

Qu, H.Q., Lawrence, S.G., Guo, F., Majewski, J., and Poly chronakos, C. (2006). Strand bias in complementary single nucleotide polymorphisms of transcribed human sequences: evidence for functional effects of synonymous polymor phisms. BMC Genomics **7,** 213 218.

Sharp, P.M., Averof, M., Lloyd, A.T., Matassi, G., and Peden, J.F. (1995). DNA sequence evolution: the sounds of silence. Philos Trans R Soc Lond B Biol Sci **349,** 241 247.

Suh, Y., Atzmon, G., Cho, M.O., Hwang, D., Liu, B., Leahy, D.J., Barzilai, N., and Cohen, P. (2008). Functionally significant insulin like growth factor 1 receptor mutations in centenari ans. Proc Natl Acad Sci USA **105,** 3438 3442.

Tsai, K.L., Sun, Y.J., Huang, C.Y., Yang, J.Y., Hung, M.C., and Hsiao, C.D. (2007). Crystal structure of the human FOXO3a DBD/DNA complex suggests the effects of post translational modification. Nucleic Acids Res **35**, 6984 6994.

Wang, F., Marshall, C.B., Yammanoto, K., Li, G.Y., Plevin, M.J., You, H., Mak, T.W., and Ikura, M. (2008). Biochemical and structural characterization of an intramolecular interaction in FOXO3a and its binding with p53. J Mol Biol **384,** 590 603.

Willcox, B.J., Donlon, T.A., He, Q., Chen, R., Grove, J.S., Yano, K., Masaki, K.H., Willcox, D.C., Rodriguez, B., and Curb, J.D. (2008). *FOXO3a* genotype is strongly associated with human longevity. Proc Natl Acad Sci USA **105,** 13987 13992.

Zhou, H., and Hickford, J.G.H. (2008). Clonal polymerase chain reaction single strand conformational polymorphism analysis: an effective approach for identifying cloned sequences. Anal Biochem **378,** 111 112.

Zhou, H., Hickford, J.G.H., and Fang, Q. (2006). A two step procedure for extracting genomic DNA form dried blood spots on filter paper for polymerase chain reaction amplifica tion. Anal Biochem **354,** 159 161.

Address correspondence to:
Jon G.H. Hickford, Ph.D.
Department of Agricultural Sciences
Faculty of Agricultural and Life Sciences
Lincoln University
PO Box 84
Lincoln 7647
New Zealand

E-mail: jon.hickford@lincoln.ac.nz

Received for publication December 5, 2010; received in revised form January 5, 2011; accepted January 6, 2011.

ANIMAL GENETICS Immunogenetics, Molecular Genetics and Functional Genomics

doi:10.1111/j.1365-2052.2009.01982.x

No association between variation in the ovine calpastatin gene and either longevity or fertility in sheep

S. O. Byun*, H. Zhou*, C. M. Frampton† and J. G. H. Hickford*

*Department of Agricultural Sciences, PO Box 84, Lincoln University, Canterbury 7647, New Zealand. †Christchurch School of Medicine and Health Sciences, PO Box 4345, Christchurch, New Zealand

Accepted for publication 28 August 2009

Veterinary Immunology and Immunopathology 130 (2009) 272–274

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Technical Report

Development of a simple typing method for the ovine Toll-like receptor 4 gene

S.O. Byun, H. Zhou, J.G.H. Hickford *

Gene-Marker Laboratory, Cell Biology Group, Agriculture and Life Sciences Division, PO Box 84, Lincoln University, Canterbury, New Zealand

ARTICLE INFO

Article history:
Received 1 July 2008
Received in revised form 11 December 2008
Accepted 17 December 2008

Keywords:
Toll-like receptor 4 gene (*TLR4*)
Polymorphism
Sheep
PCR-SSCP

ABSTRACT

Toll like receptor 4 (TLR4) recognizes lipopolysaccharide (LPS) from Gram negative bacteria, as well as a number of other ligands. Genetic variation in the TLR gene has been associated with altered immune responses to pathogens and results in variation in disease susceptibility. The objective of this work was to develop a simple and rapid genotyping system for ovine TLR4 and of a sensitivity that would allow detection of allelic variation in this gene. While variation in exon 3 of the ovine TLR4 gene has been described previously, we describe here an improved genotyping method. This method could not only reveal the four alleles that have been reported previously, but also revealed a further three new alleles of this gene in a population of 1670 New Zealand sheep. This improved genotyping method will be useful in understanding innate immune responses in individual sheep and could also be a useful tool for large scale immune studies in sheep production systems.

Analytical Biochemistry 385 (2009) 174–175

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Notes & Tips

An effective method for silver-staining DNA in large numbers of polyacrylamide gels

S.O. Byun, Q. Fang, H. Zhou, J.G.H. Hickford *

Gene-Marker Laboratory, Agriculture and Life Sciences Division, Lincoln University, Lincoln 7647, New Zealand

ARTICLE INFO

Article history:
Received 31 August 2008
Available online 25 October 2008

ABSTRACT

Silver staining of nucleic acid has been used for various biological analyses, including polymerase chain reaction single strand conformation polymorphism (PCR SSCP) analysis. A variety of methods have been described, but these methods are not that effective for staining more than a few PCR SSCP gels, especially rapidly and with high sensitivity, because they include a number of time consuming or hazardous manual steps that are often time dependent. Here we report a silver staining method that can efficiently stain up to 14 gels at one time and with a detection limit of approximately 10 pg of DNA/mm^2, which is comparable to other methods.

0003-2697/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2008.10.024

Mol Biotechnol (2009) 41:133 137
DOI 10.1007/s12033 008 9103 2

RESEARCH

Haplotypic Diversity Within the Ovine Calpastatin *(CAST)* Gene

S. O. Byun · H. Zhou · J. G. H. Hickford

Published online: 20 September 2008

doi:10.1111/j.1365-2052.2008.01745.x

Removed due to copyright

Association of the ovine *calpastatin* gene with birth weight and growth rate to weaning

S. O. Byun*, H. Zhou*, R. H. J. Forrest*, C. M. Frampton† and J. G. H. Hickford*

*Agriculture and Life Sciences Division, PO Box 84, Lincoln University, Canterbury 7647, New Zealand. †Christchurch School of Medicine and Health Sciences, PO Box 4345, Christchurch 8140, New Zealand

Accepted for publication 27 March 2008

doi:10.1111/j.1365-2052.2008.01720.x

Lack of association between *CAST* SNPs and meat tenderness in sheep

H. Zhou*, S. O. Byun*, C. M. Frampton†, R. Bickerstaffe* and J. G. H. Hickford*

*Gene Marker Laboratory, Agriculture and Life Sciences Division, PO Box 84, Lincoln University, Lincoln 7647, New Zealand.
†Christchurch School of Medicine and Health Sciences, PO Box 4345, Christchurch, New Zealand

Accepted for publication 10 February 2008

Available online at www.sciencedirect.com
ScienceDirect
Molecular and Cellular Probes 22 (2008) 131 132
Molecular and Cellular Probes
www.elsevier.com/locate/ymcpr

Short Communication

Polymorphism of the ovine insulin-like growth factor I receptor (*IGFIR*) gene

S.O. Byun, H. Zhou, J.G.H. Hickford*

Gene Marker Laboratory, Agriculture and Life Sciences Division, P.O. Box 84, Lincoln University, Canterbury, New Zealand

Received 16 August 2007; accepted 22 August 2007
Available online 14 September 2007

Removed due to copyright

doi:10.1016/j.mcp.2007.08.007